Establishment of Long-Term Culture of Bovine Undifferentiated Germ Cells Isolated from Adult and Immature Testes

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Abstract

Cultured spermatogonial stem cells (SSCs) can be a useful tool for introducing genetic modification through gene targeting and genome editing technologies. However, the establishment of cell lines from SSCs seems to be dependent on species and the age of animals. To our knowledge, there are no reports about the culture systems of SSCs isolated from adult livestock animals. Different culture systems for bovine undifferentiated germ cells consisting SSCs isolated from both immature and adult testes were established in this study.

Firstly, culture condition for undifferentiated germ cells isolated from adult testis was developed in this study. Undifferentiated germ cells are rare in adult bovine seminiferous tubules, the isolation of these cells must be followed by enrichment procedures. Percoll gradient centrifugation was followed by plating the cell suspensions on gelatin-coated dishes. The enriched cells were cultured for the first 10 passages in the medium containing BIO and subsequently the cultures were maintained in the medium containing GDNF. Typical mouse male germ stem (GS) like cell colonies were formed under these culture conditions as recognized by their botryoidally morphology. All cell lines expressed germ-cell-specific markers such as UCHL-1, DBA, and GFRα-1 and pluripotent stem-cell-specific markers *OCT4*, *SOX2*, and *KLF4*. Germ cells from adult bovine testis were cultured for about 3 months under these conditions.

Next, culture condition for undifferentiated germ cells isolated from immature testis was also developed. Undifferentiated germ cell consisting of SSCs isolated from immature testes can give rise to germ-cell lines under serum-free culture conditions. During the early stages of culture, the colonies were identified by their botryoidally morphology. After about one month of the culture, tightly packed 3-dimensional colonies like as mouse embryonic stem (ES) cells were observed. These ES cell-like colonies were maintained for

at least 2 months. However, the aggregated botryoidally colonies were maintained for more than 3 months under serum-free culture conditions. The expression of both germ-cell-specific markers (UCHL-1 and DBA), and pluripotent stem-cell-specific markers (*OCT4*, *SOX2*, *KLF4*) were also confirmed when predominant ES-like colonies were present in the culture. When in vitro differentiation analysis was performed, cell lines formed embryonic bodies (EBs). After EBs were cultured for another 9 days, they differentiated into three germ layers confirmed by the expression of ectoderm (*NESTIN*), mesoderm (*BMP4*), and endoderm (*GATA*-6) markers by RT-PCR. Immunofluorescense analysis suggested that the cell lines differentiated into neuron-like cells confirmed by the expression of GFAP.

The stem cell potential of the established cell lines both of immature and adult testes were examined by aggregation of the cell lines with 8- to 16-cell stage embryos. Cell lines from immature testes were incorporated into the ICM region in 26.67% of blastocysts. In contrast, the contribution of cell lines from adult testes was limited.

Taken together, the evidence shows that cells of cell lines derived from immature testes have a partial stem cell potential, but those from adult testis have strong characteristic as germ cell lines and a weak potential as stem cells. This finding demonstrates that the feasibility of SSC culture in domestic species, which could facilitate progress in research related to transgenic animal production, genome editing technology for the improvement of livestock production or conservation of endangered species.

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List of Abbreviations

ANOVA	: Analysis of variance
ART	: Assisted reproductive technology
BCL6B	: B-cell CLL/Lymphoma B
BIO	: 6-bromoindirubin-3-oxamine
BMP4	: Bone marphogenetic protein 4
C-KIT	: Receptor tyrosine kinase
CDH1	: Cadherin 1
cDNA	: Complementary deoxyribonucleic acid
CFDASE	: Carboxyfluorecein diacetate succiminidyl ester
CSF-1	: Colony stimulating factor 1
DAPI	: 4',6-diamidino-2-penylindole
DBA	: dolichos biflorus agglutinin
DMEM/F12	: Dulbeco modified eagle's medium F12
EBs	: Embryonic bodies
EGF	: Epidermal growth factor
ERK1/2	: Extracelullar signal-regulated kinase 1/2
ES cells	: Embryonic stem cells
FACS	: Fluorescence activated cell sorting
FBS	: Fetal bovine serum
FGF	: Fibroblast growth factor
FITC	: Fluorescein isthiocyanate
GATA-6	: GATA binding factor-6
GDNF	: Glial cell line-derived neurotropic factor
GFAP	: Glial fibrillary acidic protein

GFRα-1	: GDNF family receptor alpha-1
GS cells	: Germ line stem cells
HE	: Hematoxylin eosin
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICM	: Inner cell mass
Id4	: Inhibitor of DNA binding 4
IGF-1	: Insulin-like growth factor 1
KSR	: Knockout serum replacement
LIF	: Leukemia inhibitory factor
LIN28A	: Lin-28 homolog A
MACS	: Magnetic activated cell sorting
MAP2K1	: Mitogen-activated protein kinase 1
MAPK	: Mitogen-activated protein kinase
MASC	: Multipotent adult spermatogonial-derived stem cells
mGS cells	: mouse germ line stem cells
MUG cells	: Male undifferentiated germ cells
NGN3	: Neurogenin-3
OCT4	: Octamer binding transcription factor 4
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
PFA	: Paraformaldehyde
PGC	: Primordial germ cell
PI3K	: Phosphoinositide 3-kinase
PLZF	: Promyelocytic leukemia zinc finger potein
POU5F1	: POU class 5 homeobox 1
RA	: Retinoic acid

RHOX10 : Reproductive homeobox 10

RNA : Ribonucleic acid

RT-PCR : Reverse transcriptase polymerase chain reaction

SALL4 : Sal-like protein 4

SCF-1 : Stem cell factor 1

SOHLH : Spermatogenesis and oogenesis specific basic helix-loop-helix 1

SOX-2 : SRY (sex determining region Y)-box 2

SSCs : Spermatogonial stem cells

STAT3 : Signal transducer and activator of transcription 3

STRA8 : Stimulated by retinoic acid 8

SYCP3 : Synaptonemal complex protein 3

TBST : Tris-buffered saline, 0.01% tween 20

TE : Trophectoderm

Thy-1 : Thymus cell antigen 1

UCHL-1 : Ubiquitin carboxyl-terminal hydrolase isozyme L1

UTF1 : Undifferentiated embryonic cell transcription factor 1

ZBTB16 : Zinc finger and BTB domain containing 16

Chapter 1: General Introduction

1.1. Spermatogonial stem cells

1.1.1. Developmental origin of spermatogonial stem cells

Mammalian zygotes are generated by fertilization of male and female germ cells. Therefore, germ cells are a unique cell type that transfer genetic information to the next generations. Primordial germ cells (PGC) are the earliest precursor of sperm and oocyte in mammal (Figure 1-1). During embryonic development, PGCs proliferate and migrate to the gonad, and then differentiate into male or female germ cells. In male gonad, PGCs begin to differentiate into their progenitor cells called gonocyte or pro-spermatogonium (Culty, 2009).

After birth, gonocytes migrate to the basal compartment of the seminiferous tubule of the testis in most mammals. According to their migratory events and mitotic activity, gonocytes can be divided into three types. The first type of gonocytes are actively multiplying gonocytes (M-pro-spermatogonium) derived from PGCs. Multiplying gonocytes give raise into primary transitional gonocyte (T1-pro-spermatogonium), which are in the quiescent stage. Transitional gonocytes differentiate into the secondary transitional gonocytes (T2-pro-spermatogonium), which migrate from the center to the periphery of the seminiferous tubule (Hilscher et al., 1974). This migratory events are promoted by transcription factor RHOX10 as one of the member of X-linked homeobox gene (Song et al., 2016). Finally, secondary transitional gonocytes give rise into SSCs as the mark of the beginning of spermatogenesis (Figure 1-1).

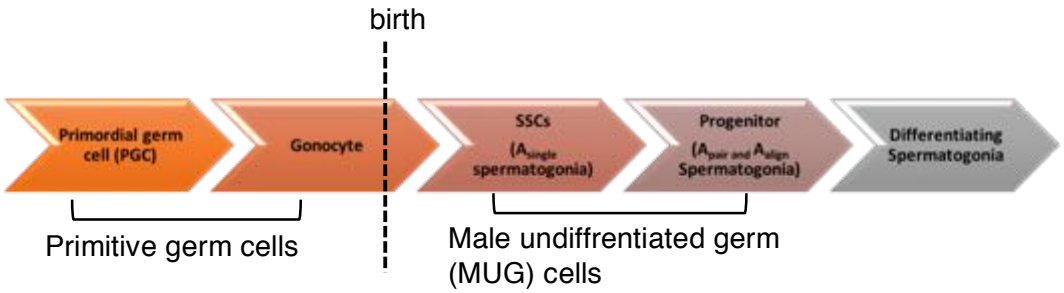


Figure 1-1. Schematic diagram of the origin of spermatogonia.

During embryonic development, PGCs differentiate into gonocytes, so called embryonic primitive germ cells. Gonocytes migrate to the basal compartment of the seminiferus tubule and initiating spermatogenesis by producing SSCs (type A spermatogonia). These mechanisms occur soon after birth in rodent, but take several weeks in domestic species. SSCs self-renew and differentiate into their progenitor. Both of SSCs and their progenitor categorize as male undifferentiated germ (MUG) cells. Finally, differentiating spermatogonia enter meiosis and spermatogenesis occur to produce mature sperm.

1.1.2. Identification of spermatogonial stem cells

Models estimating the origin of SSCs have been reported in many decades. Base on their morphology, spermatogonial cells are classified in several types such as: type A, intermediate, and type B spermatogonia. In rodent, type A spermatogonia is the common source of undifferentiated spermatogonia. They consist of A_{single} spermatogonia, A_{pairs} spermatogonia, and $A_{aligned}$ spermatogonia types (Rooij et al., 2000). In the past decades, A_{single} spermatogonia population in the testis are considered as SSCs (Oakberg, 1971) (Tegelenbosch and de Rooij, 1993). They self-renew and differentiate into their progenitor cells such as A_{pairs} and $A_{aligned}$ spermatogonia. However, further studies by transplantation assay revealed only few of A_{single} spermatogonia are SSCs (Nagano et al., 1999). Population of A_{single} spermatogonia in the testis is about 0.02-0.03% from total germ cells, however, transplantation assay estimated only 0.002% of SSCs population in the testis.

Another approach to identify SSCs is by using biomolecular markers. The known markers for mouse SSCs are GFRα-1 (Naughton et al., 2006), Thy-1 (Kubota et al., 2003), or Id4 (Sun et al., 2015), OCT4 (POU5F1/OCT3/4), PLZF (promyelocytic leukemia zinc finger; ZBTB16/ZFP145), LIN28A, BCL6B, NGN3, CDH1, UTF1, and SALL4 (reviewed by (Manku and Culty, 2015). In which, LIN28A (Chakraborty et al., 2014) and NGN3 (Nakagawa et al., 2010) mostly express in SSCs progenitor cells (Figure 1-2).

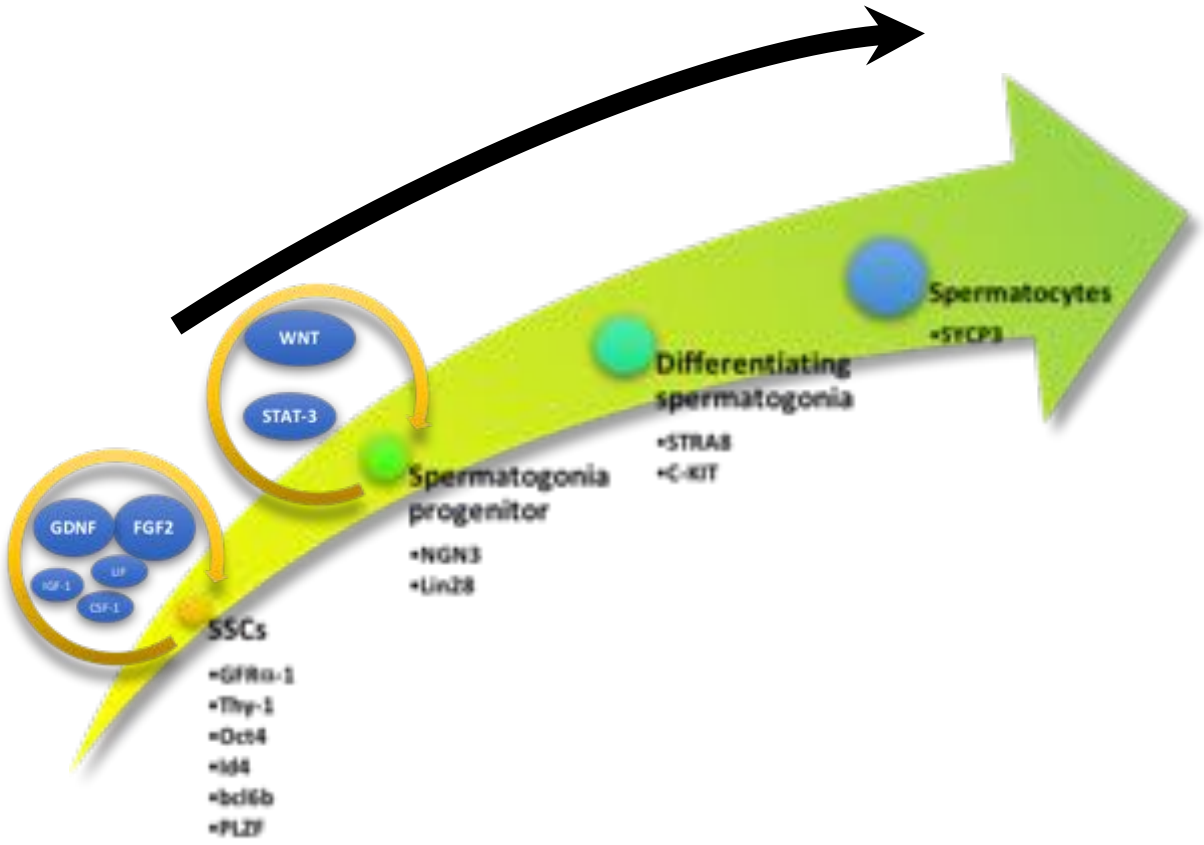


Figure 1-2. Schematic diagram of molecular signaling and growth factors during spermatogonia development in mouse.

Different types of germ cells are recognized by several biomolecular markers. GDNF, FGF2, IGF-I, LIF, and SCF-1 are the known growth factors to promote the self-renewal and proliferation of SSCs, while WNT and STAT-3 support the proliferation of progenitor cells derived from SSCs. Once SSCs/progenitors induced by retinoic acid (RA), they will start to differentiate into STRA-8-positive cells and subsequently enter meiosis phase.

1.2. Spermatogonial stem cells niche in the testis

The self-renewal activity of SSCs is rapidly occur during neonatal development (Oatley and Brinster, 2012). When animals are in adulthood, self-renewal activity of SSCs become a day-to-day event. They will follow long-term cyclic production of SSCs (Kanatsu-Shinohara et al., 2016). To maintain the stability of spermatogenesis, SSCs are supported by microenvironment "Niche" to promotes their self-renewal and differentiation in balance. SSCs are surrounded by unique features of somatic cells including Sertoli cells, Leydig cells, and myoid cells. These somatic cells secrete various growth factors to support SSCs self-renewal and differentiation (Oatley and Brinster, 2012).

1.2.1. Factors supporting spermatogonial stem cells self-renewal

One of the essential growth factors to support SSCs self-renewal is glial cell line-derived neurotropic factor (GDNF), which is secreted by Sertoli cells (Figure 1-2). In transgenic mice, over expression of GDNF is stimulated self-renewal, by contrast reduced level of GDNF expression in transgenic nice results in cell differentiation (Meng et al., 2000). Addition of GDNF to mouse SSCs culture medium leads rapid phosphorylation of Akt (Lee et al., 2007) and ERK1/2, those prevent cell differentiation of SSCs (He et al., 2008). Hence, GDNF regulates SSCs self-renewal via activation of Src-PI3K/Akt pathway and Ras/ERK1/2 pathway ((Hofmann, 2008).

Another growth factor associated with the maintenance of SSCs is fibroblast growth factor 2 (FGF2). FGF2 is secreted by various testicular cells including Sertoli cells, Leydig cells, and differentiating germ cells. FGF2 mediates SSCs self-renewal through the activation of MAP2K1 signaling pathway (Ishii et al., 2012). Addition of FGF2 to mouse SSCs culture medium promotes the phosphorylation of MAP2K1 and upregulates the expression of Etv5 and Bcl6b.

STAT3 and Wnt/ β-catenin have been also reported as a signaling pathway involved in the regulation of spermatogonia in the testis. However, whether these molecules involve

in the SSCs maintenance or differentiation is still unclear. STAT3 binds to its downstream target NGN3 in the Thy1-positive SSCs and regulates NGN3 transcription (Kaucher et al., 2012). NGN3 is a marker for SSCs progenitors. In addition, activation of Wnt/β-catenin pathway maintain stem cells characteristics of SSCs (Yeh et al., 2012) and support the proliferation of SSCs progenitor cells in mouse (Takase and Nusse, 2016).

Leukemia inhibitory factor (LIF), insulin-like growth factor I (IGF-I), and colony stimulating factor 1(Csf-1) have been reported as external growth factors for self-renewal of SSCs (Oatley et al., 2009) (Kubota et al., 2004). However, mechanisms involved in the regulation of SSCs self-renewal are poorly understood.

1.2.2. Factors for supporting spermatogonial stem cells differentiation

SSCs will undergo differentiation upon they are induced by factors secreted from somatic cells. There are few known factors involving in SSCs differentiation. Retinoic acid (RA) have been widely known as SSCs differentiation stimulating factor (Figure 1-2). Upon induced by RA, SSCs express some marker proteins differentiating spermatogonia such us STRA8, KIT, SOHLH1, and SOHLH2 (Oatley and Brinster, 2012). STRA8 is a target gene of RA induction.

In vivo condition of spermatogonia differentiation is a complex process with the involvement of multiple molecules secreted into the microenvironment in the testis. It is difficult to mimic this process in vitro. (Sato et al., 2011) reported the organ culture system for in vitro production of functional sperm in rodent, however, this method seems difficult to be applied in large animals. Recently, functional sperm can be also produced by aggregating pluripotent stem cells with embryonic testicular cells (Ishikura et al., 2016). It can be concluded that the role of testicular somatic cells in producing haploid genome is indispensable.

1.3. In vitro culture of spermatogonial stem cells

Growth factors involving in SSCs proliferation and self-renewal have been studied for several decades. Development of in vitro culture system of SSCs is useful for understanding of biological processes in male gametogenesis. As reported in a decade ago, mouse neonatal SSCs are successfully cultured for about 5 months in the presence of GDNF, epidermal growth factor (EGF), basic FGF, and LIF (Kanatsu-Shinohara et al., 2003). Interestingly, subsequent research from the same group revealed that under appropriate conditions mouse SSCs culture generate embryonic stem (ES) like cells (Kanatsu-Shinohara et al., 2004). Then followed by the establishment of multipotent stem cells generated from adult mouse SSCs (Guan et al., 2006) (Seandel et al., 2007) (Ko et al., 2009). Study about the epigenetic features of SSCs indicates its plasticity (Liu et al., 2016). Thus, in vitro culture of mouse SSCs can be established male germline stem cell (GS cells), ES-Like cells (mGS cells), and multipotent adult spermatogonial-derived stem cells (MASC) (Figure 1-3).

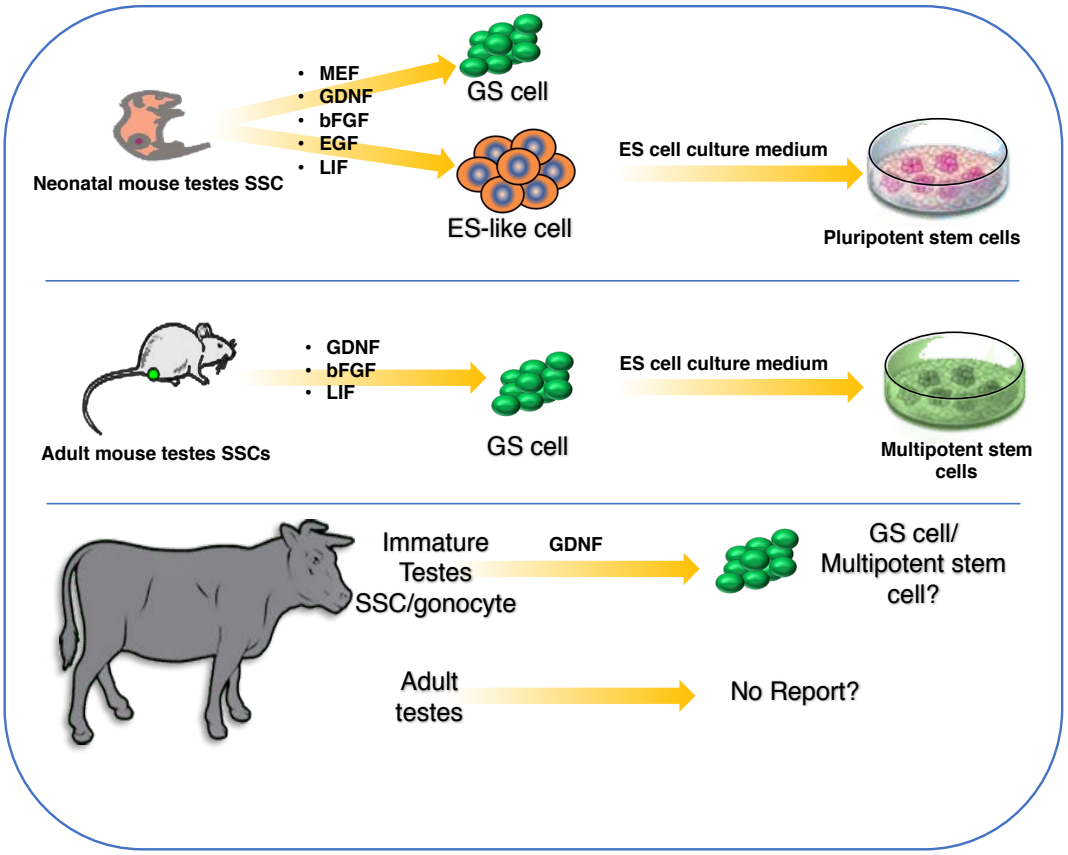


Figure 1-3. Overview of germ cell culture in mouse and cattle.

1.4. Spermatogonial stem cells in domestic animal

1.4.1. Characterization of spermatogonial stem cells

Expression of biomolecular markers in germ cells from domestic species is heterogeneous. They express in most of undifferentiated germ cells in the testis (Zheng et al., 2014), however, it is difficult to distinguish between undifferentiated germ cells and SSCs. Undifferentiated germ cells including SSCs are identified by the expression of DBA in cattle (Izadyar et al., 2002), (Fujihara et al., 2011), in pig (Goel et al., 2007), in buffalo(Goel et al., 2010)); UCHL-1 in cattle (Herrid et al., 2007),(Fujihara et al., 2011), in buffalo (Goel et al., 2010), in goat (Heidari et al., 2012)); GFRα-1 in pig (Lee et al., 2013), in cattle (Oatley et al., 2004), and in horse (Costa et al., 2012)). Another approach to characterize SSCs in domestic species is by recognizing their morphology and the localization in the testis. SSCs are located closely to the basement membrane of the seminiferous tubule.

1.4.1 Isolation of spermatogonial stem cells from the testis in domestic animal

As mentioned above, the population of SSCs in mouse testis analyzed by transplantation assay is only 0.002% in the total of testicular germ cells (Nagano et al., 1999). However, the population of SSCs in the testis in is poorly understood. Magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) can be applied in rodent, but not in domestic species due to lack of SSCs specific markers.

Mechanical cell sorting has been used to isolate SSCs in domestic species. Percoll gradient centrifugation (van Pelt et al., 1996),(Fujihara et al., 2011), differential plating system (Izadyar et al., 2002),(Goel et al., 2010) or their combination become standard approaches to purify undifferentiated germ cells in domestic species. It has been reported

55% enrichment for spermatogonia obtained from pre-pubertal buffalo testis by combining of both differential plating system and Percoll gradient centrifugation (Goel et al., 2010).

1.4.2. In vitro culture of spermatogonial stem cells in domestic animal

In vitro culture of SSCs in mammals has been performed for understanding biological characteristics of testicular germ cells and its application in biotechnology. Isolation and culture of SSCs from various species such as mouse (Kanatsu-Shinohara et al., 2004), pig (Goel et al., 2007), cattle (Fujihara et al., 2011) (Oatley et al., 2016), human (Conrad et al., 2008), primates (Langenstroth et al., 2014) have been reported.

The establishment of in vitro culture of germ cells in cattle has been only reported by the use of immature testes. When germ cells from adult bovine testes cultured in vitro, colonies only appear during primary culture, and cannot form colony after the colonies were passaged (Fujihara et al., 2011). Isolation and in vitro culture of SSCs from adult animal seems to be difficult due to variety of germ cells in the testicular cells and the lack of SSCs specific markers in domestic species.

Recent study reported the addition of glial cell line-derived neurotropic factor (GDNF) allows to activate MAPK signaling pathway supporting long-term proliferation of bovine SSCs (Sahare et al., 2015). GDNF is secreted by Sertoli cells to support SSCs maintenance in the germ cell niche in the testis (Meng et al., 2000). Nevertheless, there are limited reports about the mechanisms for supporting SSCs during in vitro culture other than MAPK signaling.

1.5. Scope of the thesis

The increasing number of world populations give some critical issues related to food security and land use. To secure the world populations every country has to improve their food production and provide land for housing. In the field of animal sciences, it will give a challenge for scientist to establish new technology that can produce more meat as

food and preserve some endangered species as the effect of human invasion in the wildlife. One of the promising way to solve this problem is the reproductive biotechnology by application of assisted reproductive technology (ART) and genome editing technology.

In rodent, SSCs can be genetically modified for producing transgenic mice (Kanatsu-Shinohara et al., 2008). This suggests that cultured male germ cells can be useful tools for making genetic modification through gene targeting technologies. Therefore, to establish a system for supporting a long-term culture of germ cells having stem cell characteristics in various species other than mice is important for applied biotechnology such as transgenic animals as human disease model, improvement of domestic animals, conservation of endangered species. To achieve these goals, long-term in vitro culture of germ cells isolated from immature and adult animal in domestic animals should be essential and important issues to study.

Chapter 2: Long-term culture system for bovine undifferentiated germ cells isolated from adult testes

2.1. Introduction

Spermatogenesis is a complex and chronological process in which diploid SSCs proceed through a series of differentiation steps to produce haploid spermatozoa (Rooij, 2001). For this ordered process testis microenvironment called niche is important for proper spermiogenesis (Oatley and Brinster, 2012). In niche, somatic cells produce signals which determine whether SSCs produce progeny cells that will either self-renew to maintain the stem cell pool or initiate differentiation yielding committed progenitors that produce sperm (Shinohara et al., 2001). Since SSCs are very rare cells in adult testis (Tegelenbosch and de Rooij, 1993), it is hard to isolate and study them and moreover to distinguish them from their undifferentiated progeny. So far there are several markers used to identified both SSCs and their undifferentiated germ cells in mouse: GFRα-1 is a receptor of GDNF (Naughton et al., 2006), Thy-1(Kubota et al., 2003) and Id4(Sun et al., 2015). In domestic species, undifferentiated germ cells including gonocytes and spermatogonia are identified by expressions of DBA(Izadyar et al., 2002) (Goel et al., 2007) (Goel et al., 2010) (Fujihara et al., 2011), UCHL-1 (Herrid et al., 2007) (Goel et al., 2010) (Heidari et al., 2012), and GFRα-1 (Oatley et al., 2004) (Costa et al., 2012) (Lee et al., 2013). Another method to recognize undifferentiated germ cells including SSCs is to make paraffin sections through the seminiferous tubules. SSCs and undifferentiated germ cells reside closely to the basal membrane.

Numerous information about SSCs and their undifferentiated progeny come from culture system for mouse testicular cells. It has been shown that cultured mouse neonatal SSCs can give raise to cell lines and contribute to chimeric embryos including germ cells (Seandel et al., 2007) . Moreover, genetically modified cultured SSCs produced transgenic animals (Kanatsu-Shinohara et al., 2008). This suggests that cultured undifferentiated male germ cells could be a useful tool for making genetic modification through gene targeting and genome editing technologies. However, most culture systems have been established

only in mice. In domestic species including cattle, the information about a long-term culture is very limited.

Because of the great promise of germ cell lines for biotechnology and agriculture, there is lots of interest to derive undifferentiated male germ cell lines in domestic species. It has been shown that bovine germ cells isolated from neonatal to 3 months old animals can be maintained for long-term (Izadyar et al., 2002), (Aponte et al., 2006), (Fujihara et al., 2011) (Sahare et al., 2016) (Oatley et al., 2016) in culture with added GDNF. In the testis, GDNF is produced by Sertoli cells and stimulates proliferation of SSCs and their undifferentiated progeny (Meng et al., 2000) (Hasegawa et al., 2013). In vitro study reported that the addition of GDNF activates a mitogen-activated protein kinases (MAPK) signaling pathway thus supporting long-term proliferation of bovine SSCs (Sahare et al., 2015). However, when the neonatal culture system was applied to adult bovine germ cells, the germ cells could not be maintained for a long time (Fujihara et al., 2011). Similar results were obtained with cryopreserved adult bovine spermatogonia cultured in medium containing GDNF (Cai et al., 2016).

In mice, it has been shown that an activation of Wnt/β-catenin pathway maintains stem-cell characteristics of SSCs and supports their proliferation (Takase and Nusse, 2016)'(Yeh et al., 2012). Wnt/β-catenin signaling pathway in vitro does not act directly on SSCs, but mainly on undifferentiated progenitor cells (Takase and Nusse, 2016). RNA sequencing analysis of SSCs collected from three shrew also revealed that Wnt/β-catenin signaling pathway was active in SSCs during in vitro culture (Li et al., 2016b).

The Wnt/β-catenin signaling pathway maintains the self-renewal of mouse embryonic stem (ES) cells (Sato et al., 2009). The pathway can be activated by the inhibition of GSK3 using small-molecule inhibitors such us 6-bromoindirubin-3′-oxime (BIO) (Sato et al., 2004). The effect of GSK3 inhibitors on the self-renewal and proliferation of bovine SSCs, however, is still unknown. In this report, we investigated the effect of BIO on cultured undifferentiated germ cells isolated from adult bovine testis. Our results show for

the first time that adult bovine undifferentiated germ cells proliferated in culture leading to derivation of cell lines. This culture system might be useful for livestock production, but also could be useful for conservation of endangered species, and the development of research in human fertility restoration.

2.2. Materials and Methods

2.2.1. Isolation of germ cells from bovine testis

Germ cells were isolated by sequential enzymatic digestion methods as previously reported with minor modifications (Fujihara et al., 2011). Briefly, the testes were collected from Japanese Black Cattle of the following ages; 3 months old, 1 to 2 years old, and 17 years old bulls. The collected testes were placed immediately into a tube, and covered with Dulbecco's modified Eagle's medium (DMEM)/F12 medium (GIBCO BRL Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU mL^{-1} penicillin (Sigma-Aldrich, St Louis, MO, USA), 50 mg mL^{-1} streptomycin (Sigma-Aldrich), 40 mg mL^{-1} gentamicin sulfate (Sigma-Aldrich) and 15 mM HEPES (Wako, Osaka, Japan). The testes were transported to the laboratory at 4ºC to 10 ºC within 24 h. To prepare cell suspensions, decapsulated testicular tissues were minced and treated by a three-step enzymatic digestion method. The cells were incubated with a first collagenase solution for 50 min at 37ºC, then washed three times with DMEM/F12. This was followed by the incubation with a second collagenase solution for 50 min at 37ºC and the cells washed again three times with DMEM/F12. The obtained suspensions were centrifuged and the resulting cell pellets were incubated with a third collagenase solution for only 10 min at 37ºC, washed three times and filtered through a 50-µm nylon mesh.

The filtered cell suspensions were enriched for undifferentiated germ cells by two step purification protocols. As the first method, we used a Percoll gradient centrifugation as previously reported with minor modifications (Fujihara et al., 2011). We used 60%, 40%, and 20 % Percoll gradients (GE Healthcare Life Sciences, Sweden). Cell suspensions were

loaded on the top of Percoll gradients and centrifuged at 3,000 rpm for 30 min. After centrifugations, different Percoll fractions were observed and the purity was assessed by immunofluorescence staining for DBA and UCHL-1. We collected only the bottom fraction of 20% Percoll and called it as 20% enriched fractions. Then we collected from half to the bottom of 40% Percoll fraction and called it as a 40% enriched fractions. All other Percoll fractions were not further used. In order to remove further remaining somatic cells from Percoll fractions, we plated cell suspensions on 0.1% pre-gelatinized dishes in DMEM/F12 media supplemented with 5% fetal bovine serum (FBS, Invitrogen) for 2 h. After 2 h, most somatic cells were attached while undifferentiated germ cells were floated. We collected floated cells and used them for further studies. The purity of cell suspensions was assessed by immunofluorescence staining for DBA and UCHL-1.

2.2.2. Culture of adult bovine germ cells

The cell suspensions enriched for undifferentiated germ cells were cultured at cell density 5 x 10^4 cells per cm^2 on poly-L-lysine (0.01%) pre-coated wells in 24 well-plates (Nunc) in a basic culture medium. The basic medium consisted of DMEM/F12 supplemented with 15 % KSR (Invitrogen) and 1% of FBS. The proliferation of germ cells was studied using four different culture media; the basic medium only, the basic medium supplemented with 20ng/ml GDNF (Peprotech), the basic medium supplemented with 6-bromoindirubin-3'-oxime (BIO, 2 μM), or the basic medium supplemented with both GDNF and BIO. After 6 to 7 days of the primary culture multicellular grape-like colonies were observed in all cultures. We passaged all cultures and counted these cultures as the first passage. Cells were cultured at 37ºC, in a humidified atmosphere with 5% CO_2. Culture media were changed every 3 days.

2.2.3. Immunohistochemistry and immunofluorescences of germ cells

For immunohistochemistry, isolated testes were fixed with Buin's solution and processed as describe previously (Kim et al., 2014). The paraffin sections were

deparafinized, rehydrated and autoclaved in 10 mM citric acid buffer (Wako) for 10 min at 110°C and incubated with appropriate primary and secondary antibodies.

Immunofluorescence of freshly isolated or cultured cells were performed as described previously (Kim et al., 2014). Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized, blocked, and incubated with appropriate primary and secondary antibodies.

The following antibodies and dilutions were used DBA conjugated with fluorescein isothiocyanate (FITC) (1 : 200; Vector Laboratories, Burlingame, CA, USA), GFRα-1 (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and UCHL-1 (PGP 9.5; 1:200; Santa Cruz Biotechnology). All primary antibodies were incubated overnight at 4^0C. Then cells were washed three times with TBST (Sigma) and incubated with appropriate secondary antibodies either Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen), or Alexa Fluor 594 rabbit-anti goat (Invitrogen) for 1 h at room temperature, washed three times with TBST. Nuclei were counterstained with Hoechst 33342 (Sigma) and mounted in 50% glycerol. Samples were observed under an immunofluorescence microscope (BX 50; Olympus, Tokyo, Japan).

2.2.4. RT-PCR analysis

RNA was isolated from either freshly recovered testes or the cultured cells using Trizol reagent (Ambion, Austin, TX, USA) according to the manufacturer's instruction. cDNA synthesis was performed using 1 µg of total RNA per 20 µL PCR reaction mixture. Briefly, oligo (dT) primers were added to the isolated RNA, incubated for 5 minutes at 65°C. ReverTra Ace (Toyobo, Tokyo, Japan) were added to the RNA mixture for reverse transcriptase reaction. Samples were incubated for 60 min at 42°C followed by 5 min at 99°C. Collected cDNA were stored at -20°C or immediately used for PCR reaction. PCR amplification was performed using 1 µL cDNA per 20 µL PCR reaction mixture containing 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 x PCR buffer, 5 pmol of each primer and 1U

Table 2-1. Primer sequences used in this experiment.

Gene name	Primer sequence (5' - 3')	
	Forward	**Reverse**
UCHL-1	ACCCCGAGATGCTGAACAAAG	CCCAATGGTCTGCTTCATGAA
OCT4	AGAGAAAGCGGACGAGTAT	AGTACAGAGTAGTGAAGTGAGG
SOX2	TTACCTCTTCTTCCCACTCC	TTCTTGCTGTCCTCCATTTC
KLF4	CCCACACAGGTGAGAAACCT	ATGTGTAAGGCGAGGTGGTC
NESTIN	TGGAGCAGGAGAAACAAGGC	TCTTCAGAAAGGTTGGCACAG
BMP4	CAGCATGTCAGGATTAGCCG	GGCTTTGGGGATACTGGAAT
GATA-6	CACCACGACCACCACTTTG	ATACAGCCCGTCTTGACCTG
C-KIT	GACCTGGAGGACTTGCTGAG	AGGGGCTGCTTCCTAAAGAG
STRA8	GATGGGAATGCAAACAGCTT	GTCCAGGAAACTTGCCACAT
SYCP3	TGACTTTGTTCCAGCAGTGG	ACTTTCGGACACTTGCCATC
β-ACTIN	TCCCTGGAGAAGAGCTACGA	ACATCTGCTGGAAGGTGGAC

Taq DNA polymerase (ExTaq; TaKaRa, Tokyo, Japan). The primer sequences used for the amplification of specific genes are listed in Table 2-1. The PCR products were separated and visualized on 2% (w/v) agarose gels containing ethidium bromide.

2.2.5. Karyotype analysis

Karyotype analysis was performed using a previously described protocol (Campos et al., 2009). Cultured cells were incubated in 0.1 μg/ml KaryoMAX Colcemid Solution (Invitrogen) for 3 hours. The cells were dissociated to a single-cell suspension using 0.25% trypsin-EDTA solution. Metaphase spreads were prepared by incubating single-cell suspensions in a prewarmed (37°C) hypotonic solution (KCl 75 mM) for 15 minutes and fixed in carnoy's fixative solutions overnight at 4°C. Chromosomes were stained with VECTASHIELD mounting medium with DAPI (Vector) and observed under an immunofluorescence microscope (BX 50; Olympus, Tokyo, Japan). Thirty metaphase spreads from three independent cell lines were counted.

2.2.6. Statistical Analysis

The differences between groups of the experiments in single means comparison were analyzed using student t-test. Multiple comparison analysis was carried out using ANOVA continued by Least Significant Difference analysis.

2.3. Results

2.3.1. Expression of SSCs markers in adult bovine testis

Testis sections were prepared from both immature and adult testes. Some sections were stained with hematoxylin and eosin (HE) or processed for immunofluorescense staining. The HE staining showed that in immature testes most of undifferentiated germ cells resided closely to the basement membrane (Fig. 2.1). However, in the adult testis undifferentiated germ cells were located away from the basal compartment closer to the lumen of seminiferous tubule.

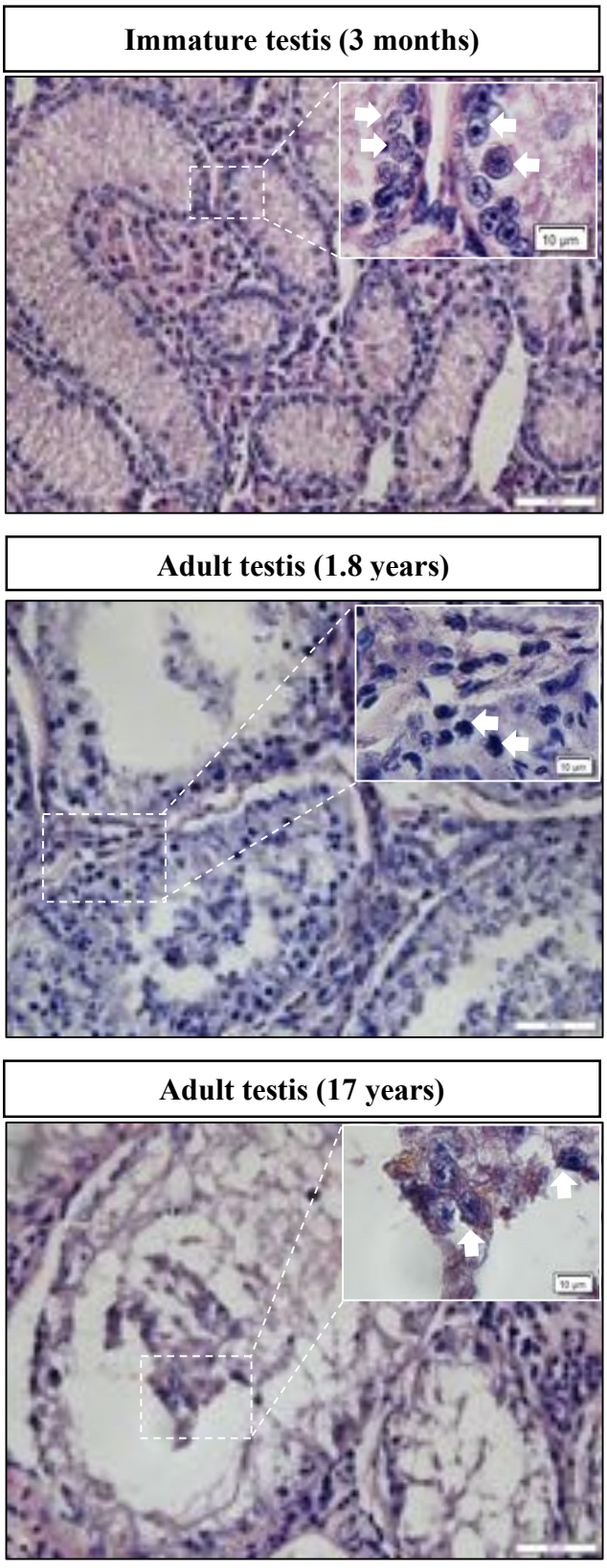


Figure 2-1. Histochemical analysis of the testis.

Hematoxylin and Eosin staining show germ cells in the seminiferous tubule of immature (3 month old) and adult (1.8 years and 17 years old) testes.

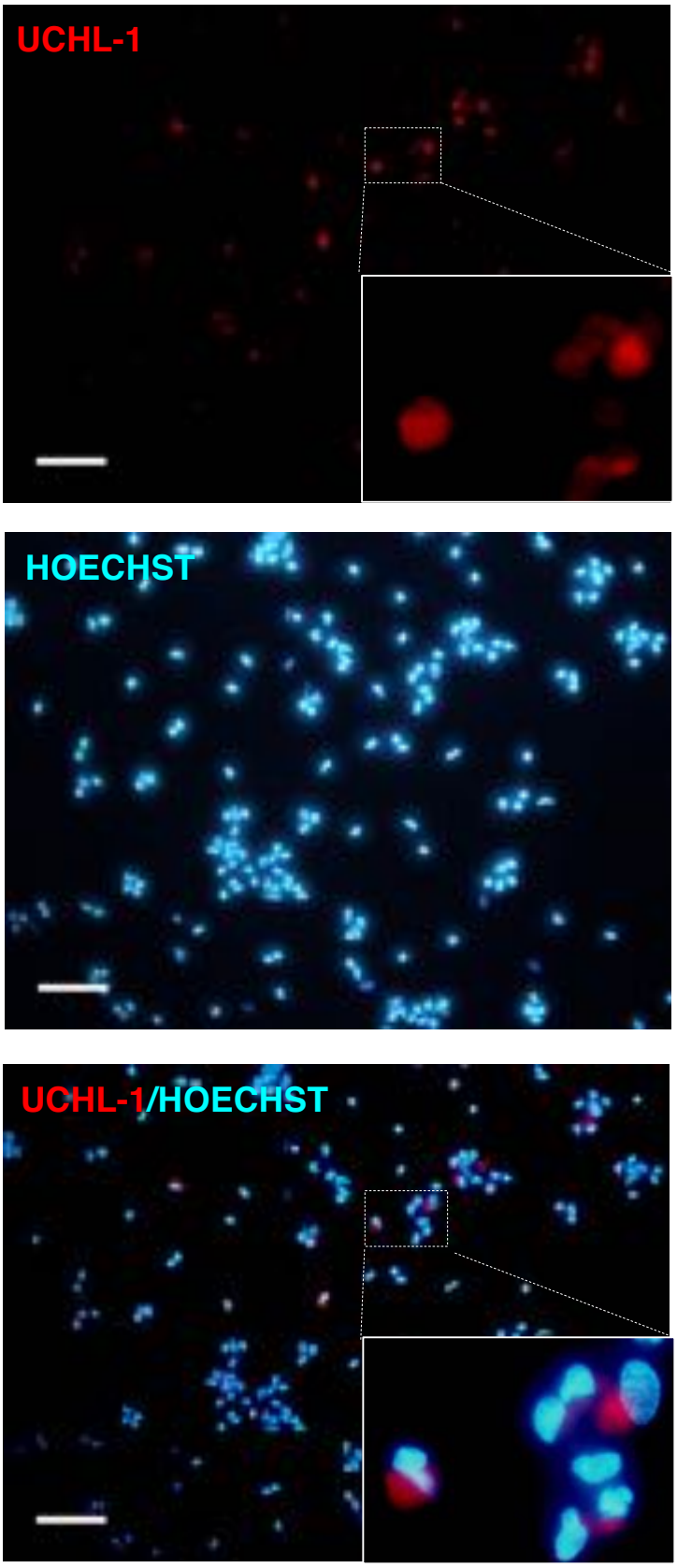
2.3.2. Enrichment for undifferentiated germ cells isolated from adult testes

Testicular cell suspensions prepared from adult bovine testis were subjected to three-step enzymatic digestion followed by two step purifications (Percoll gradient enrichment and gelatin-coated dish selection). Two fractions containing undifferentiated germ cells were collected after Percoll gradient enrichment (20 and 40 % fraction) and seeded on pre-gelatinized dishes for 2 h to remove contaminated somatic cells. DBA-positive cells were identified in both the 20% and 40% fractions. In the preliminary experiments, the DBA-positive cells recovered from the 20% fraction proliferated and formed colonies which could be further expanded after primary culture. In contrast, DBA-positive cells recovered from the 40% Percoll fraction did not form colonies and could not be maintained after primary culture. Thus, the further experiments focused on only 20% Percoll fractions.

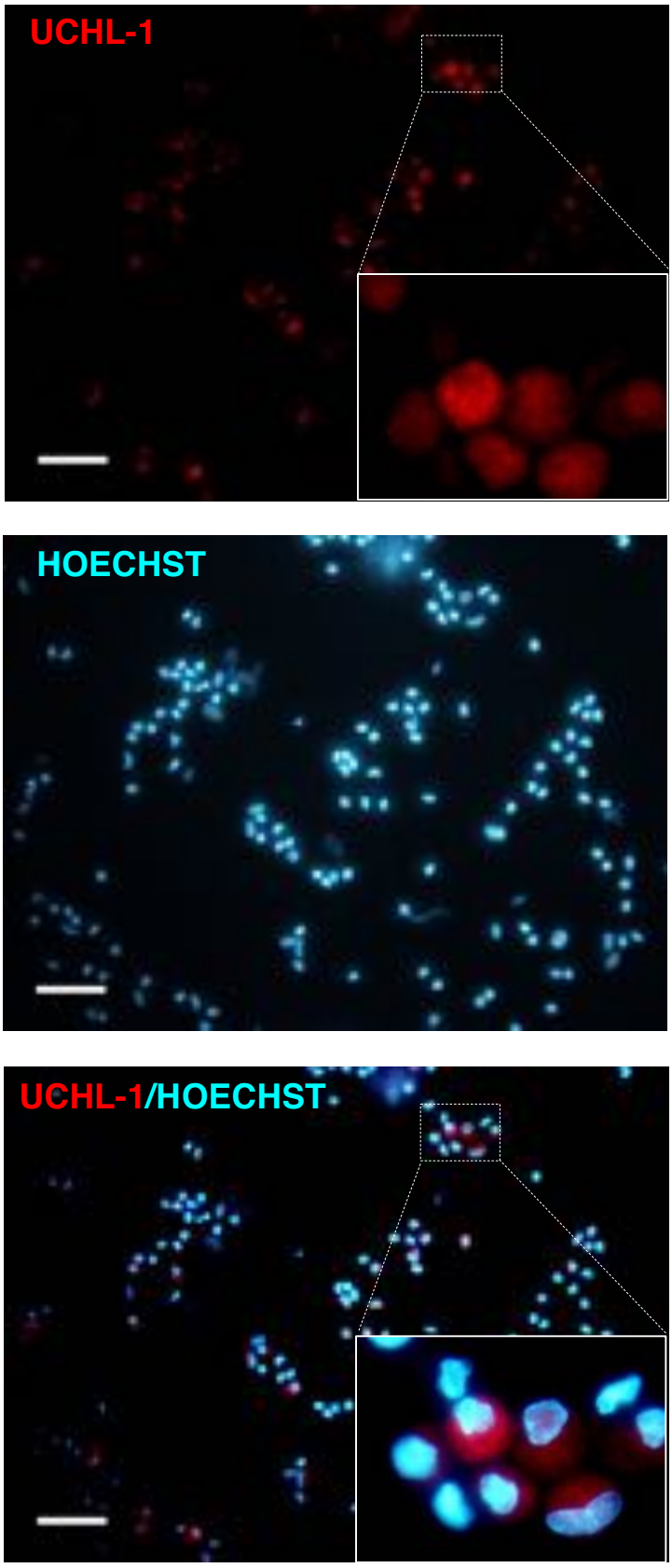
The number of undifferentiated germ cells were estimated by UCHL-1 and DBA staining (Fig. 2.2 A-D). The percentage of UCHL-1-positive cells in the cell suspensions was 10.03±3.22% and significantly increased to 27.71±10.46% after Percoll enrichment. Further enrichment for UCHL-1 positive cells was achieved by the negative selection on the gelatin-coated dishes (36.81±11.52%). A similar results were obtained by using DBA antibody (before Percoll, 15.31±2.87%; after Percoll, 52.70±7.50%; and after plating on gelatin-coated dishes, 63.62±4.60%) (Fig. 2.2E).

The same enrichment protocol was employed to enrich cell suspensions prepared from immature testes. The number of GFRα-1-positive cells was higher in immature testes (37.61± 4.94%) than in adult (7.14±3.1%) (n=6) (Fig. 2.3 A, B, and E). The same result was observed using UCHL-1 antibody (Fig. 2.3 C, D, and F). Immunostaining results were also confirmed by RT-PCR (Fig. 2.3 G).

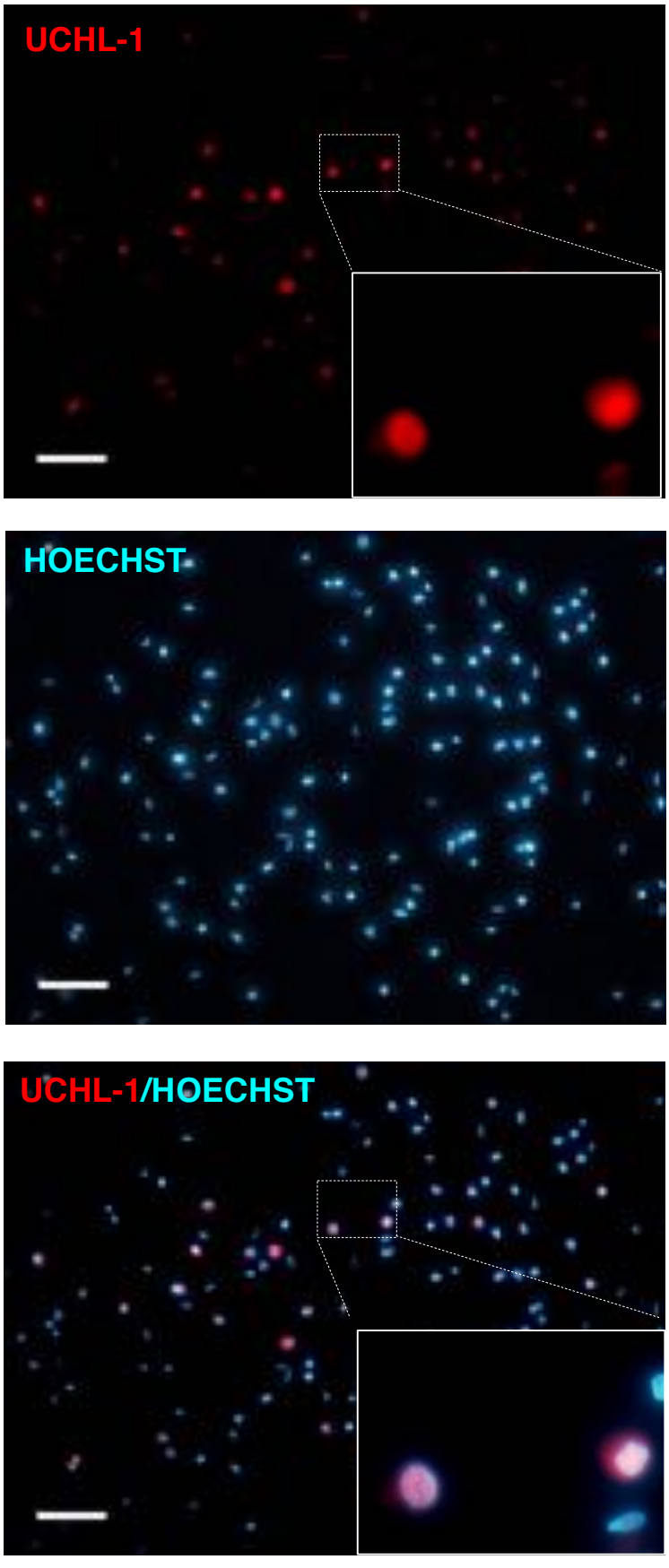
A



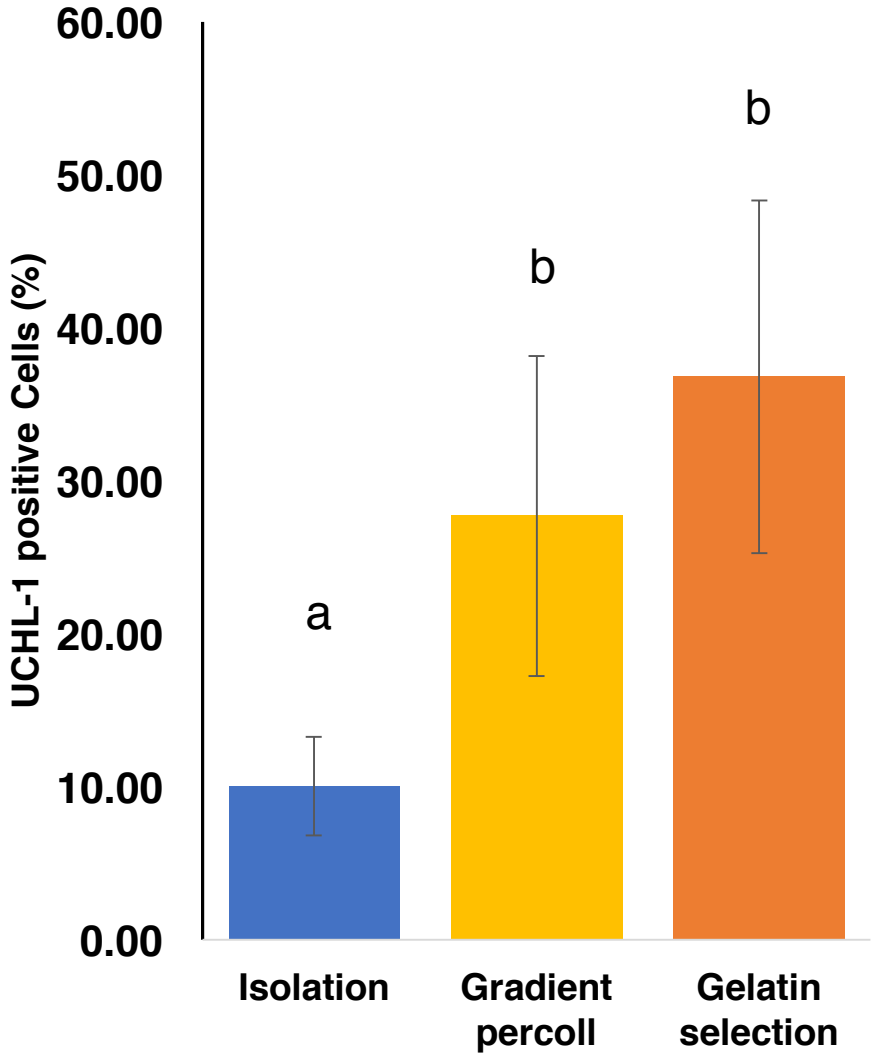
B



C



D


60.00
50.00
40.00
30.00
20.00
10.00
0.00
UCHL-1 positive Cells (%)
a
b
b
Isolation
Gradient percoll
Gelatin selection

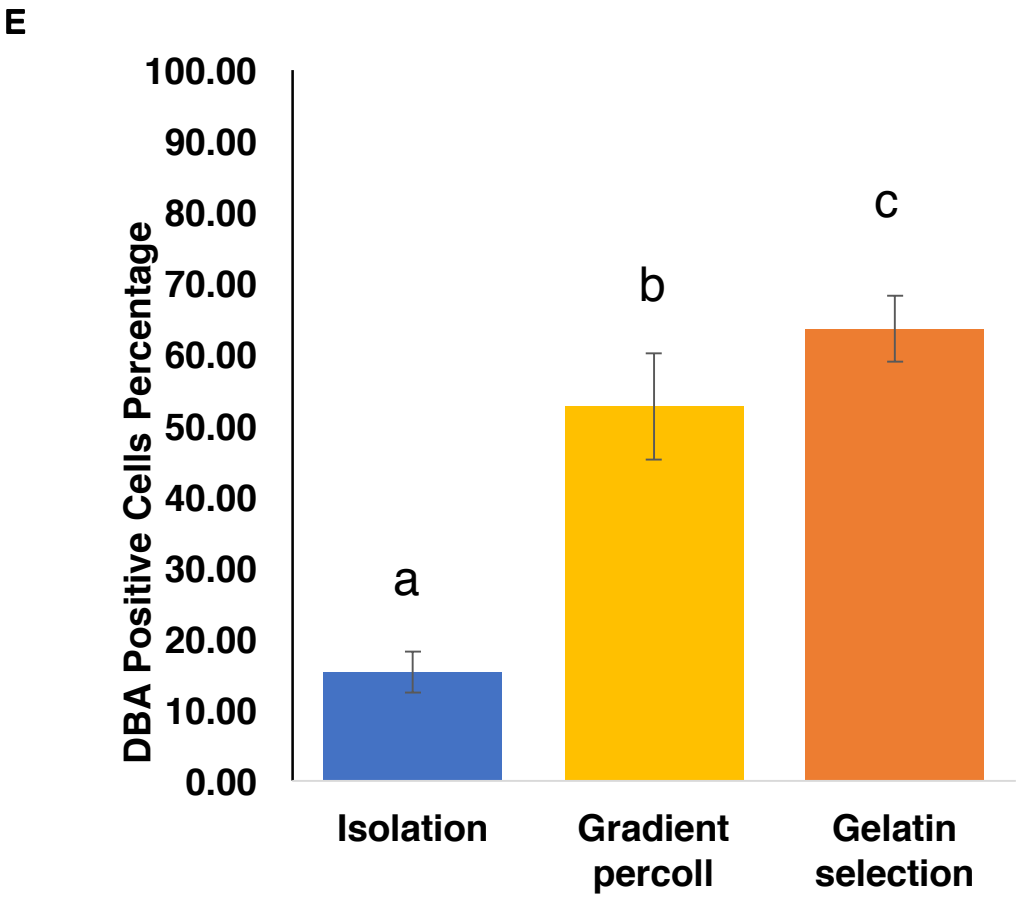
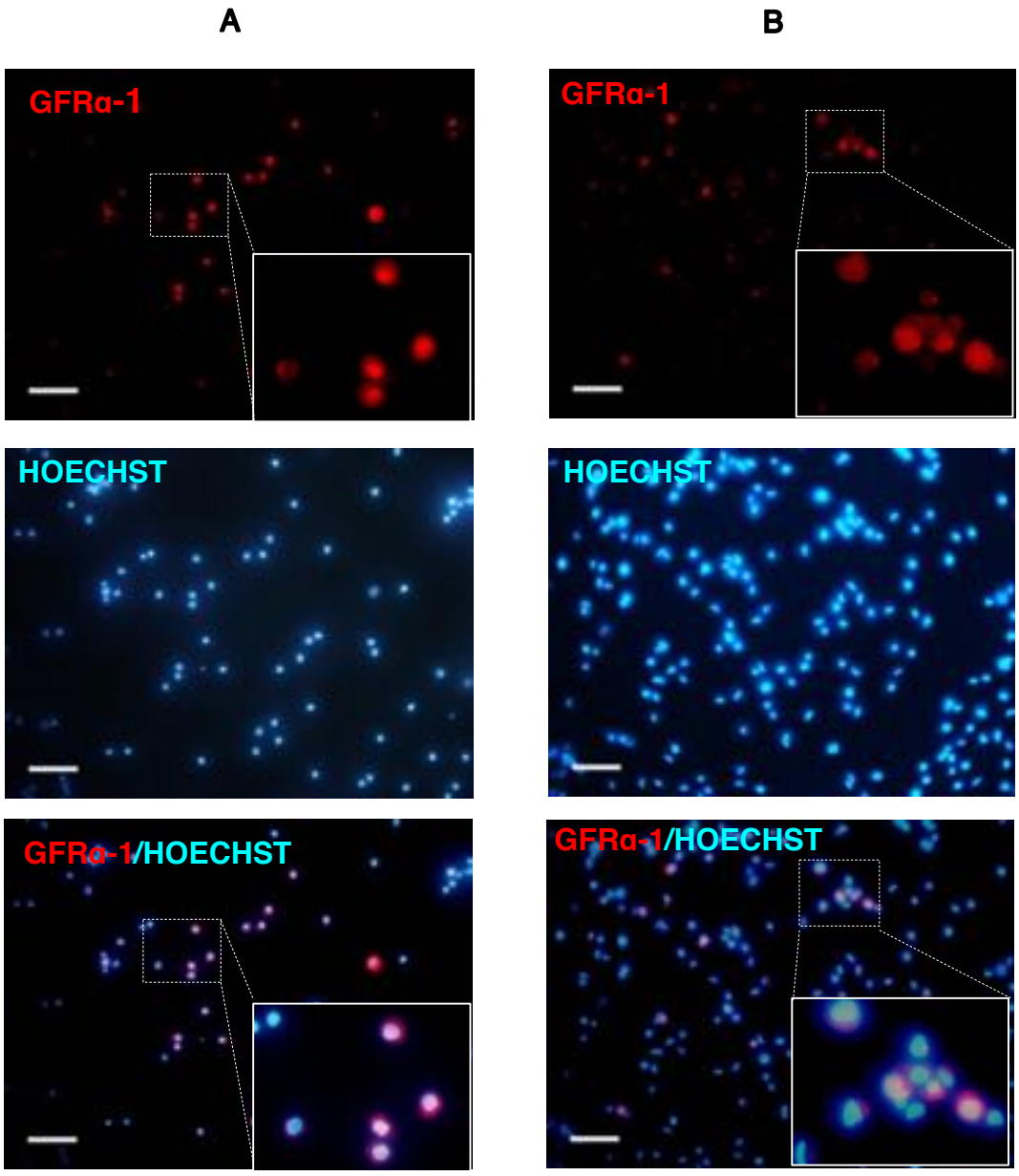
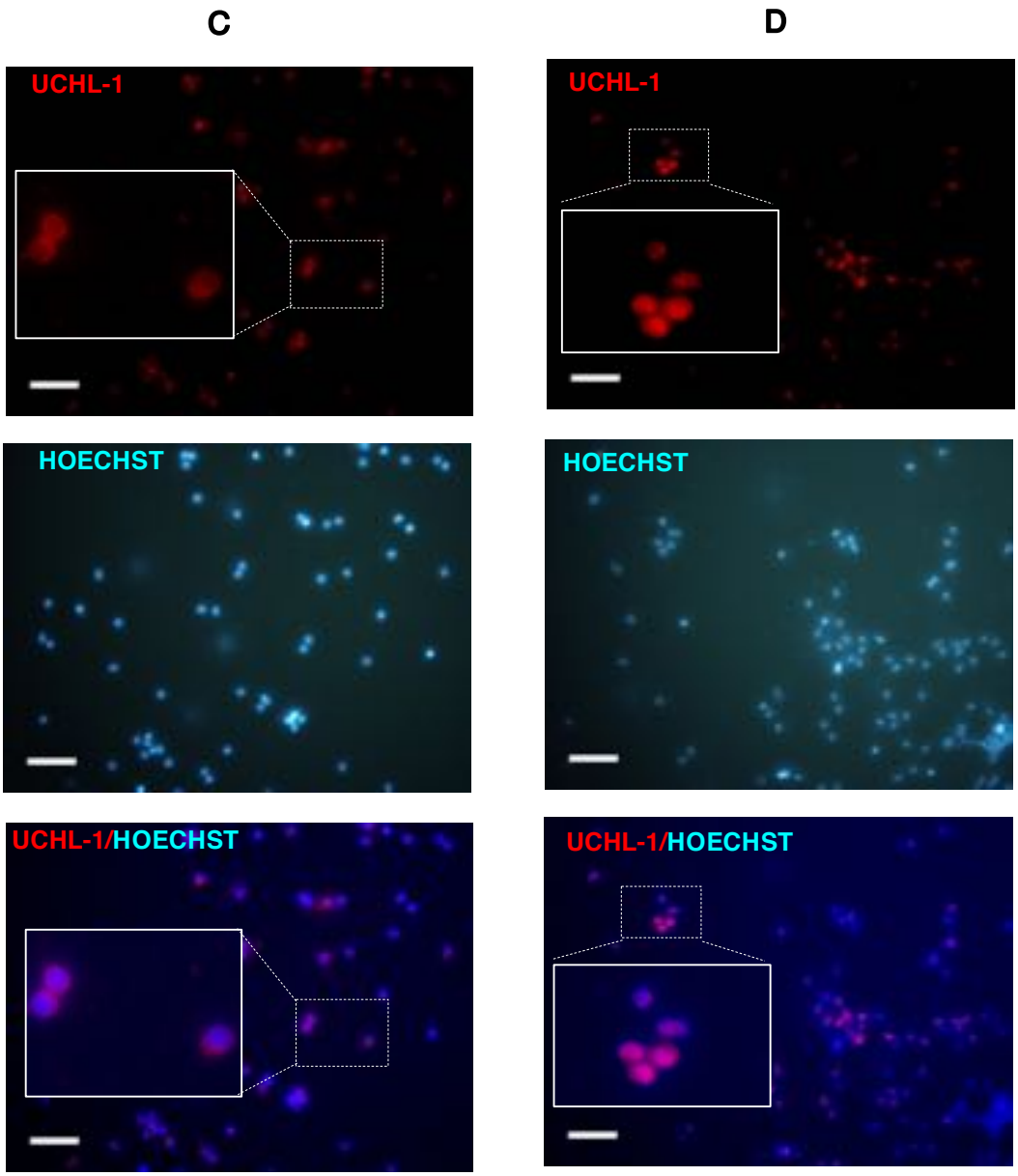


Figure 2-2. Enrichment for undifferentiated germ cells isolated from adult testes.

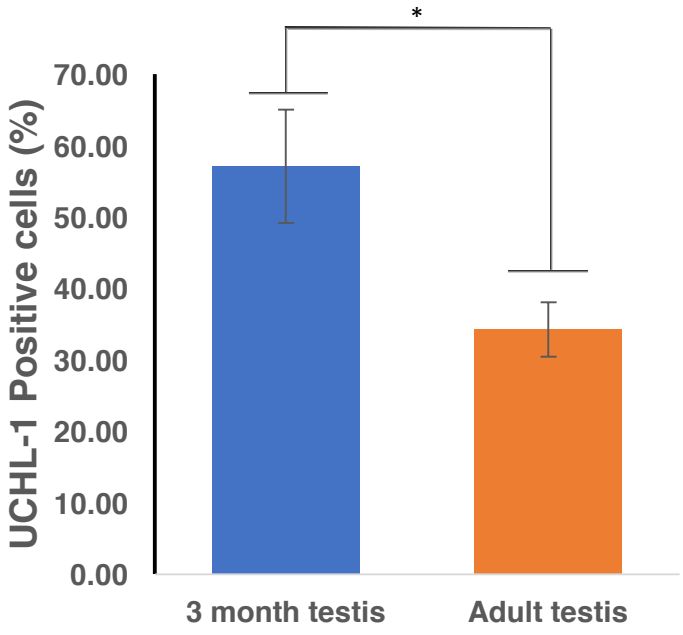
(A) - (E) show UCHL-1 positive cells in freshly isolated cells suspensions. (A) UCHL-1 positives cells in the 20% Percoll fraction, (B) UCHL-1 positives cells in the 40% Percool fraction, (C) UCHL-1 positives cells after gelatin selection. Enrichment (%) for undifferentiated germ cells was estimated either as (D) the number of UCHL-1 positive cells, or (E) DBA positive cells. Higher magnifications are shown in the

inset. Scale bars represent 50 mm and n=4.

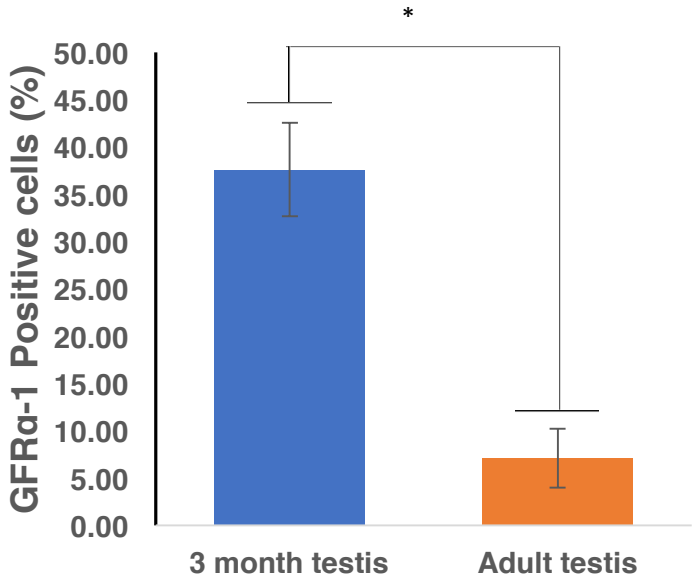

A
B
GFRα-1
GFRα-1
HOECHST
HOECHST
GFRα-1/HOECHST
GFRα-1/HOECHST


C
D
UCHL-1
UCHL-1
HOECHST
HOECHST
UCHL-1/HOECHST
UCHL-1/HOECHST

E


*
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
UCHL-1 Positive cells (%)
3 month testis
Adult testis

F


*
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
GFRα-1 Positive cells (%)
3 month testis
Adult testis

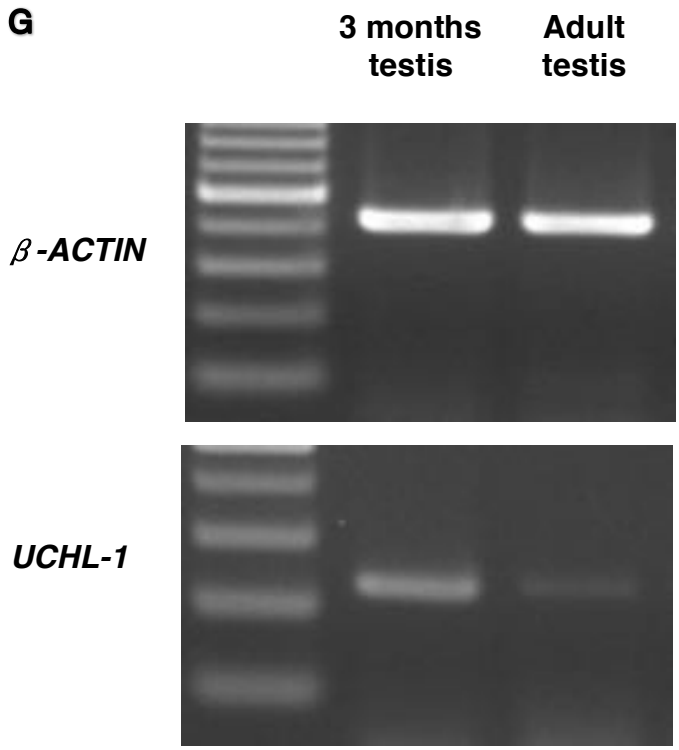
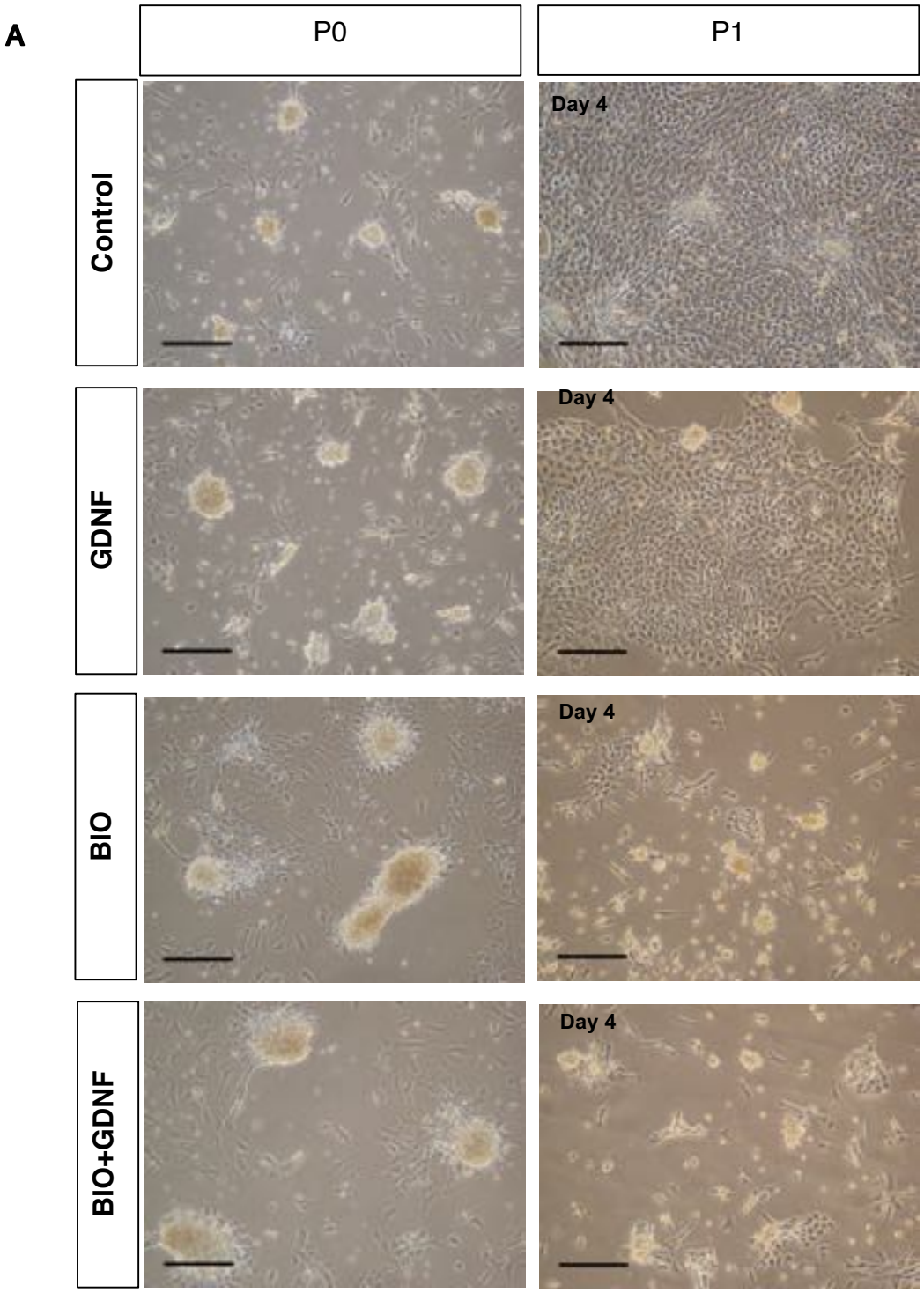


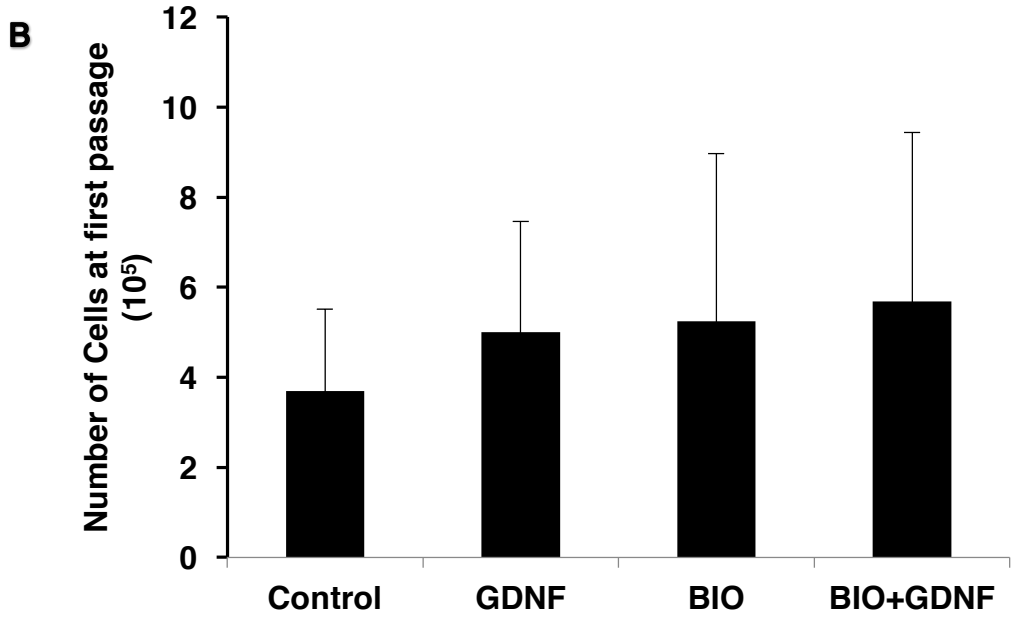
Figure 2-3. Differential expression of GFRa-1 and UCHL-1 in undifferentiated germ cells isolated from immature and adult testes.

GFRa-1 positive germ cells isolated from (A) 3 months old bovine testis and from (B) adult bovine testes. UCHL-1positive germ cells isolated from (C) 3 months bovine testis and from (D) adult bovine testis. The percentage of (E) GFRa-1 positive cells from 3 months and adult bovine testis and (F) of UCHL-1 positive cells from 3 months and adult bovine testis. RT-PCR analysis of *UCHL-1* expression in germ cells isolated from 3 months or adult bovine testis (G). Higher magnifications are shown in the inset. Scale bars represent 50 mm and n=6.

2.3.3. Colony formation of cultured adult undifferentiated germ cells

Purified undifferentiated germ cells were cultured in four different media; the basic medium, the basic medium supplemented with GDNF only, the basic medium supplemented with BIO only, or the basic medium supplemented with both GDNF and BIO. After 7 days of the primary culture, we observed grape-like shape colonies. All cultures could be passaged, however, the grape-like colonies were observed mainly in the medium containing only BIO or GDNF and BIO. In the basic medium and only GDNF containing medium, enhanced proliferation of somatic cells was observed and caused overgrowth covered on grape-like shape colonies (Fig. 2.4 A). On the other hand, the number of germ cells and germ cell specific marker expression were notably increased in the BIO and the BIO and GDNF cultures (Fig. 2.4 B and C), therefore further experiments were done in the culture in the presence of only BIO.


A
P0
P1
Control
GDNF
BIO
BIO+GDNF
Day 4
Day 4
Day 4
Day 4



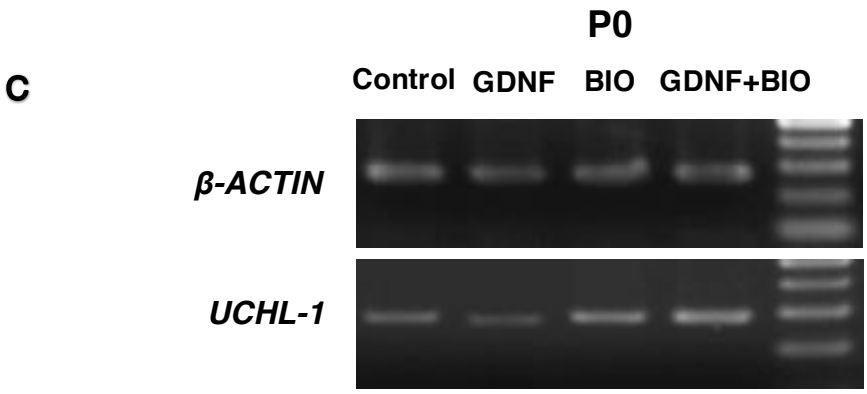


Figure 2-3. Colony formation of adult undifferentiated germ cells cultured under different conditions.

Undifferentiated germ cells isolated from adult testes were cultured in the basic medium in the presence of GDNF, BIO, or BIO and GDNF. (A) Colonies in the primary culture (P0) or after the first passage (P1). (B) The number of germ cells was counted after the first passage. (C) RT-PCR analysis of UCHL-1 expression (germ cell markers) on colonies cultured in different culture conditions. Scale bars represent 100 mm

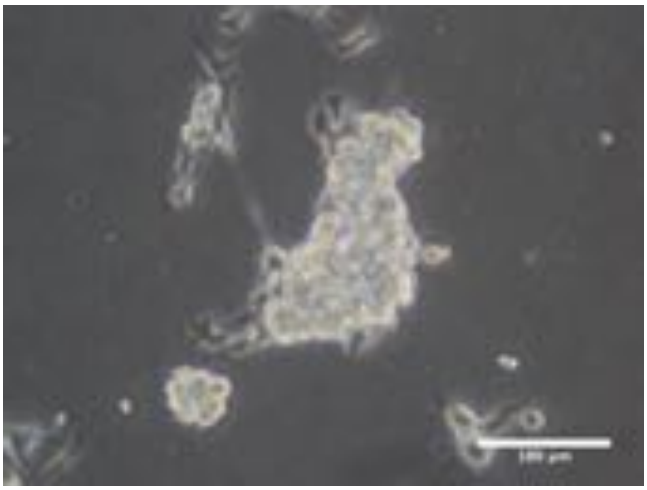
2.3.4. Characterization of long-term cultured cell lines

Typical germ cell colonies were maintained in the presence of BIO (Fig. 2.5 A). RT-PCR analysis was performed on the colonies at passages 3, 8 and 10 showed that the germ-cell-specific marker *UCHL-1* was stably expressed, and the stem-cell specific markers *OCT4* and *KLF4* were also detected with a slightly different expression pattern in *SOX2* (Fig. 2.5 B). Moreover, immunofluorescence analysis confirmed the expression of DBA and GFRα-1 (Fig. 2.5 C).

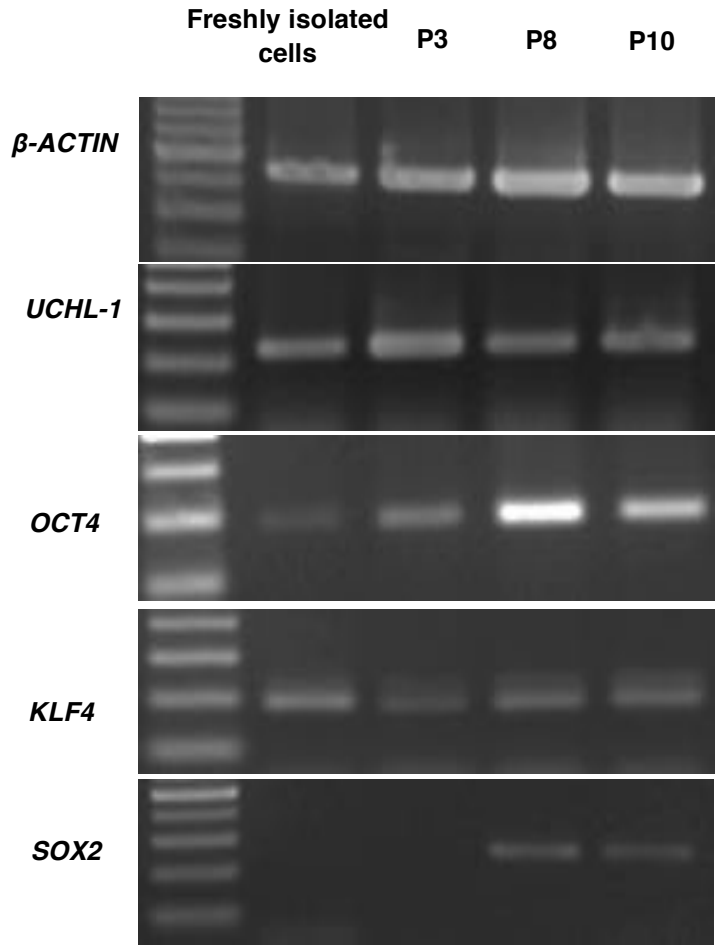
We hypothesized that once adult undifferentiated germ cells were reprogrammed to self-renew in the presence of BIO, the BIO could be removed and replaced by GDNF. We removed BIO at passage 4, 5, 6, or 10 and replaced by GDNF at each stage. Only cultures kept with BIO in culture for 10 passages and then the replace of BIO by GDNF could maintain colony formation and give rise to a long-term culture of germ cells (Fig. 2.6 A). Total 3 cell lines from 9 adult testes were established and were maintained for more than 3 months. These cell lines had grape-like shape morphology and expressed both DBA and GFRα-1 germ cell markers (Fig. 2.6 B).

Established 3 cell lines expressed undifferentiated germ cells specific markers UCHL-1 at the passage 18 (Fig. 2.6 C). We also compared the expression of differentiating germ cells specific markers *C-KIT*, *STRA8*, and *SYCP-3* between freshly isolated germ cells from adult testis and cultured germ cells at passage 18 by RT-PCR. *C-KIT, STRA8*, and *SYCP-3* were expressed in isolated germ cells from adult testis as expected, but only c-kit was expressed in cultured germ cells (Fig. 2.6C). Moreover, *VIMENTIN* was also detected in these cultures.

A



B


Freshly isolated cells
P3
P8
P10
β-ACTIN
UCHL-1
OCT4
KLF4
SOX2

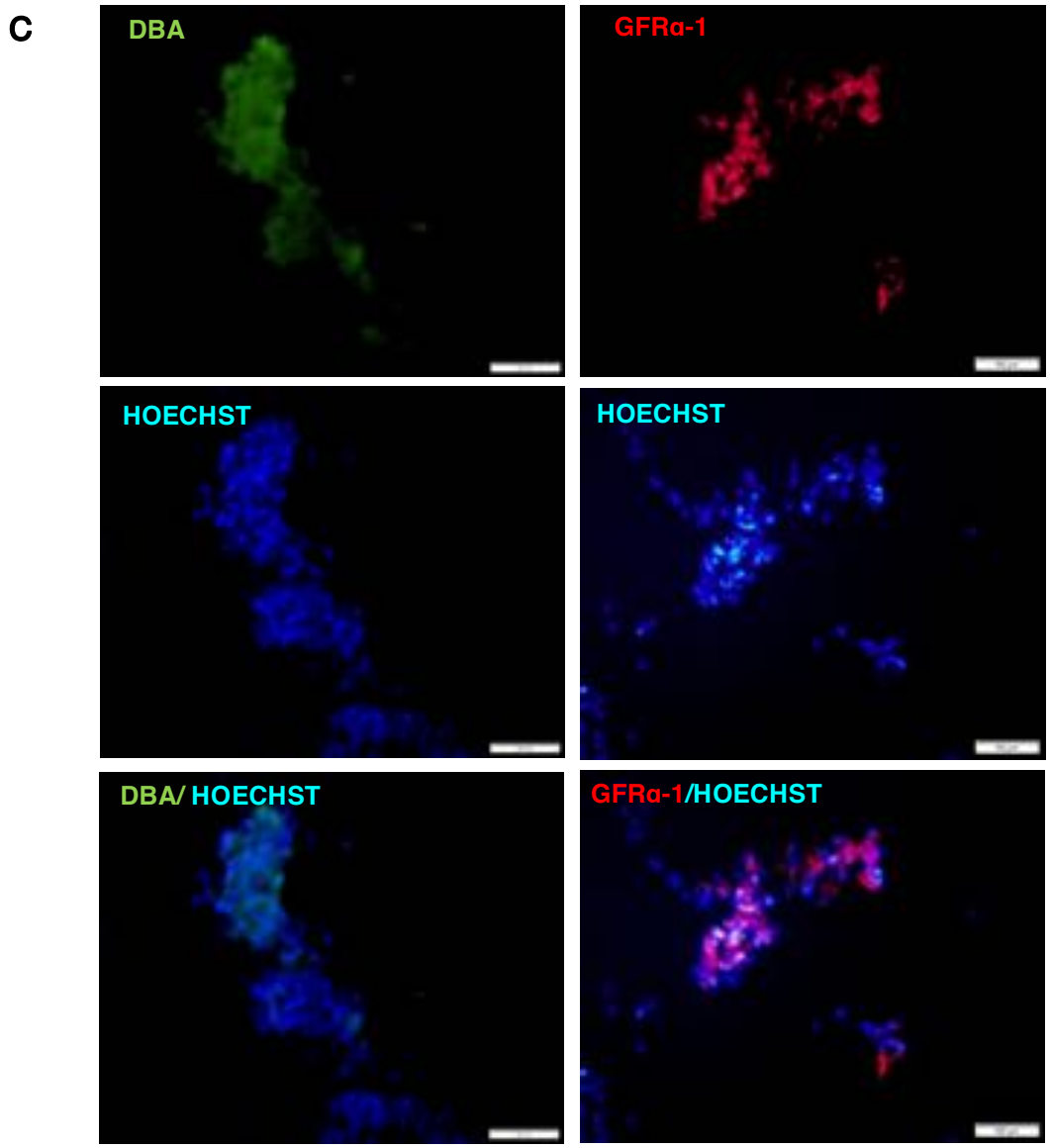
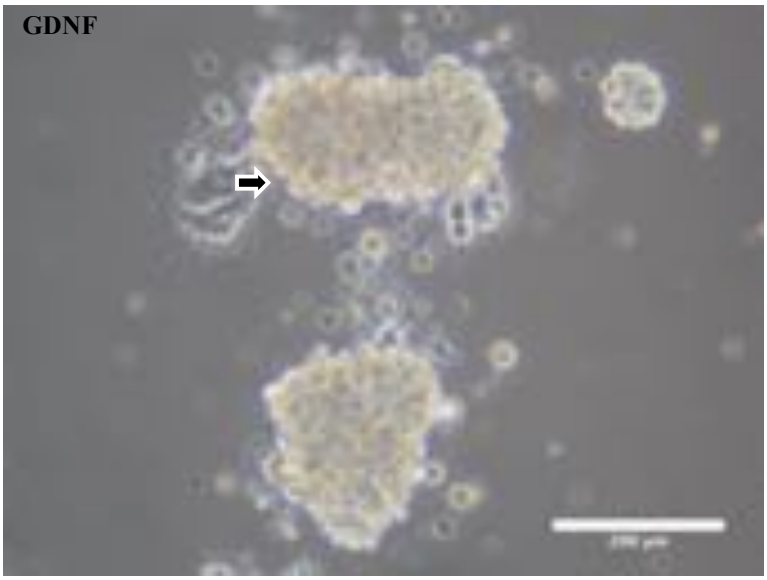


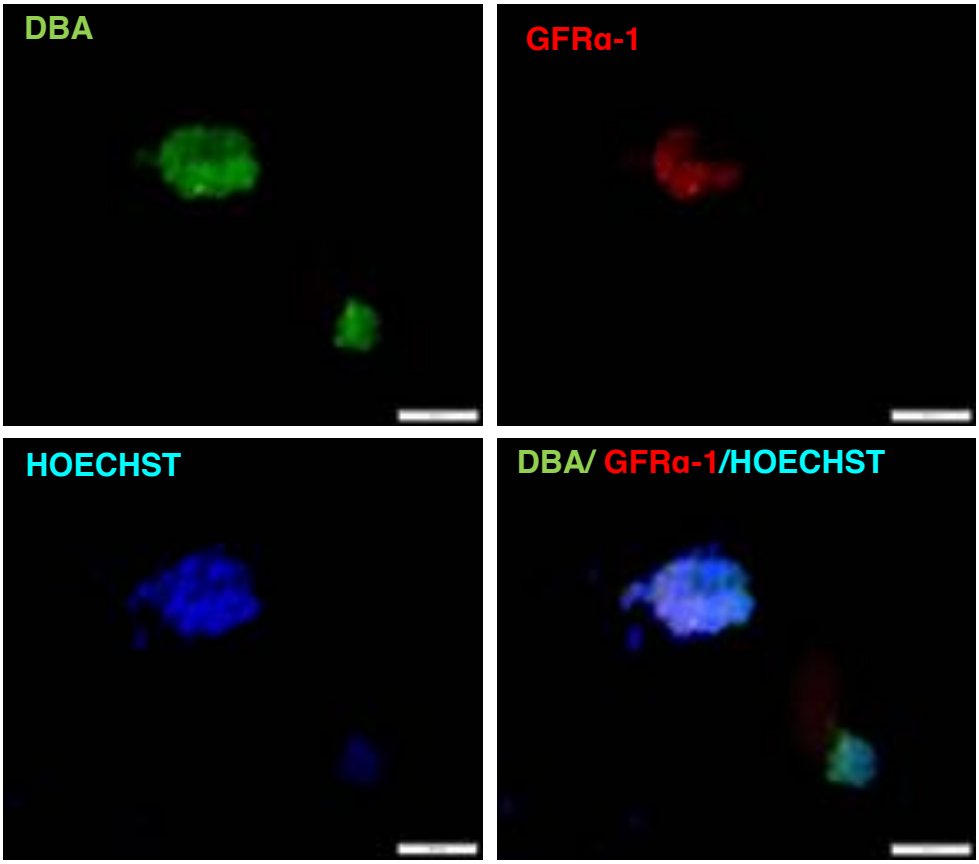
Figure 2-4. Characterization of germ cell colonies in the presence of BIO.

(A) A typical germ cell colony at the passage 10 (P10). (B) RT-PCR analysis of UCHL-1, OCT3/4, and KLF4, and SOX2 at P3, P8, and P10. (C) Immunofluorescence expression of DBA and GFRα-1.

A



B



C

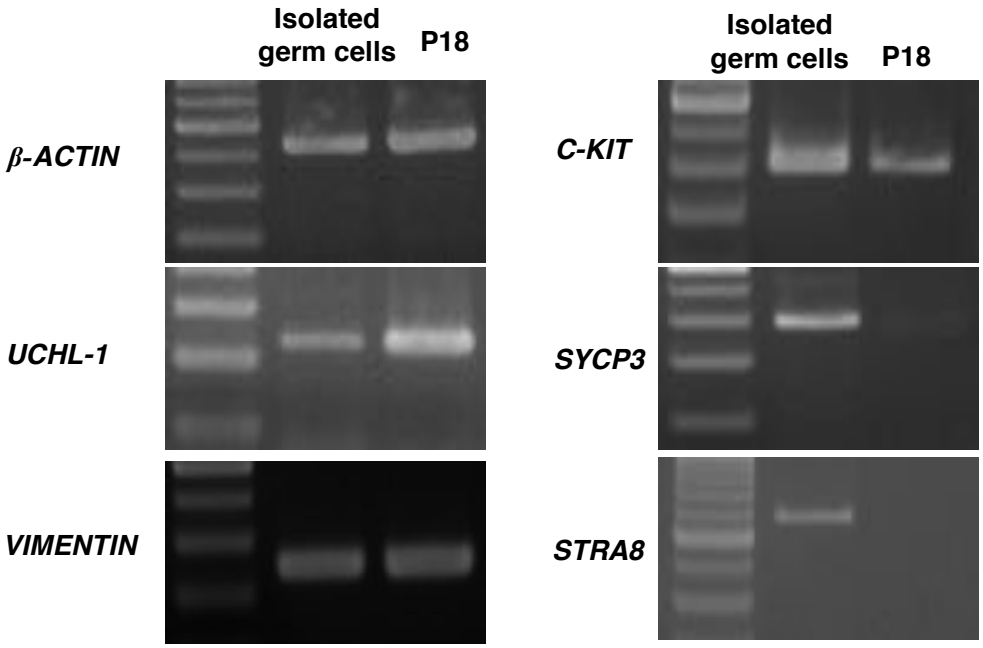


Figure 2-5. Morphology and immunofluorescence analysis of established cell lines from adult testis in the presence of GDNF.

A grape-like shape morphology of colonies in the presence of GDNF (A) Immunofluorescences analysis of DBA and GFRa-1 (B), and RT-PCR analysis of *UCHL-1, C-KIT*, *STRA8*, and *SYCP-3* expressions at P18 (C).

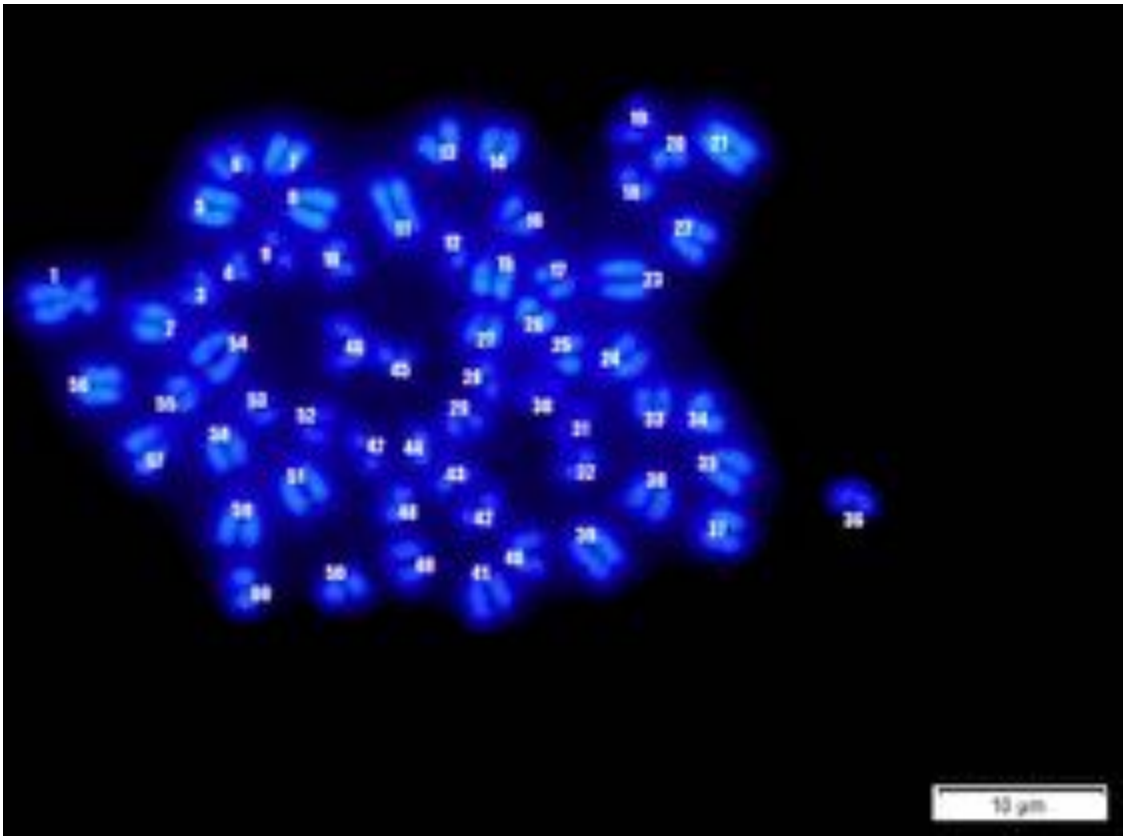


Figure 2-6. Karyotyping image of germ cell lines from adult testis.

Karyotyping analysis was performed on the cell lines at passage 14 and had the normal number of chromosome (2n=60).

2.3.5. Karyotype analysis of cell lines from SSCs of immature and adult bovine testes

Karyotype analysis also showed normal metaphase spreads and chromosome number (2n=60) in germ cell lines from adult testes (96.7%, 29 out of 30 spreads), and 3.33% had 59 chromosomes (Fig. 2.7).

2.4. Discussions

In this work, a successful derivation of adult bovine undifferentiated cell lines is reported. Since SSCs and their undifferentiated progeny are rare in adult seminiferous tubules, cell suspensions collected from the testis are necessary to enrich the population of undifferentiated germ cells. Fluorescence activated cell sorting (FACS) (Guan et al., 2006) and magnetic activated cell sorting (MACS) (Gassei et al., 2009) are the most common methods used to purify undifferentiated germ cells in mouse and rat. However, both techniques are difficult to use in domestic species due to limited knowledge about species specific markers for undifferentiated germ cells. Therefore, different approaches in domestic species have been used to purify male undifferentiated germ cells including SSCs. Percoll gradient centrifugation (van Pelt et al., 1996)·(Fujihara et al., 2011), differential plating system (Izadyar et al., 2002)·(Goel et al., 2010) or the combination of these methods become standard approaches to purify undifferentiated germ cells. Spermatogonia obtained from pre-pubertal buffalo testis are enriched 55% by combining of both differential plating system and Percoll gradient centrifugation (Goel et al., 2010).

In this experiment, enriched germ cell fractions from adult bovine testis were prepared by applying two enrichment steps. Percoll gradient centrifugation for the first step

was performed and then negative selection of germ cells to remove testicular somatic cells on gelatin coated dishes was followed. Two Percoll fractions (20% and 40%) were significantly enriched for DBA-positive cells, however, the germ cells from 40% Percoll fraction did not form colonies after the primary culture. These results suggested that DBA positive cells recovered from 40% Percoll fraction were either differentiating or already differentiated germ cells. Similar observations were made previously by Fujihara et al. (2011). Therefore, germ cells collected from 20% Percoll fractions were used for further experiments.

Enriched cell suspensions were cultured in four different culture media. Germ cells cultured in the basic medium or medium supplemented with GDNF proliferated and formed grape-like shape colonies during the primary culture. However somatic cells also proliferated and eventually overgrown superior to germ cells colonies in the secondary cultures. In contract, cultures supplemented with both GDNF and BIO or BIO only supported germ cell proliferation and colony formations even after the first passaged.

Previous report (Sahare et al., 2015) showed that bovine undifferentiated germ cells isolated from immature testis can be maintained for a long-term in the presence of GDNF. Therefore, we hypothesized that once adult undifferentiated germ cells have an ability stem cell-like properties and/or reprogrammed to SSCs characteristics in the presence of BIO, then BIO can be omitted from the culture and replaced by GDNF. When BIO was removed earlier at the 10 passage from the cultures and then replaced by GDNF, the grape-like colonies were formed. This result suggests that during first 10 passages in BIO, some of the undifferentiated adult germ cells were reprogrammed into germ cells similar to immature germ cells. Consistent with our results, the recent study in tree shrew indicates that Wnt/β-catenin signaling pathway is involved in the maintenance of adult germ-cells during the early stage of in-vitro culture (Li et al., 2016b) .

In our culture system, undifferentiated germ cells could be passaged as single cells after trypsin-EDTA treatment. However, the resulting colonies after the passage were

closely associated with somatic cells. In order to determine the origin of somatic cells, we performed by RT-PCR by the expression of *VIMENTIN*, a marker of Sertoli cells. The importance of Sertoli cells in germ-cell cultures have been recently reported in several species including mouse (Mäkelä et al., 2014), pig (Wang et al., 2015), goat (Pramod and Mitra, 2014), and cattle (Nasiri et al., 2012) (Cai et al., 2016). Moreover, Sertoli cells secrete various types of substrates including GDNF in the germ-cell niche (Hofmann, 2008) (Meng et al., 2000).

Established cell lines after passage 18 and freshly isolated adult germ cells were characterized by RT-PCR for *c-KIT* (marker of differentiating spermatogonia (Wang et al., 2016)), and *STRA-8* and *SYCP-3* (markers of both differentiated germ cells or early meiotic germ cells (Wang et al., 2016)). *c-KIT*, *STRA-8* and *SYCP-3* were expressed in freshly isolated adult germ cells. In contrast, only *c-KIT* was detected in analyzed cell lines (Fig.2-6 C). This suggested that undifferentiated germ cells were mostly maintained in our culture system. However, undifferentiated germ cells expressing *UCHL-1* did not express *OCT-4* at the passage 18. The significance of the presence of OCT-4 protein in germ cells of domestic species is poorly understood. OCT-4 is expressed in gonocytes and germ cells residing near to the basement membrane, however, OCT-4 expression was weak in spermatogonia in adult bovine testis (Fujihara et al., 2011).

Our culture system gave rise to 3 cell lines derived from 9 adult testes. Germ cells isolated from 17 years old sire cultured for at least one month, but could not maintain the cells further. The 17 years old sire had already finished semen collection for artificial insemination, which may result in a quiescent condition in sperm production.

Chapter 3

Long-term culture system for bovine undifferentiated germ cells isolated from immature testes

3.1. Introduction

Male undifferentiated germ (MUG) cells consist of spermatogonial stem cells (SSCs) and their progenitors. In mammalian species, SSCs are one of stem cells in the testis and carry genetic information to the next generation. It has been shown that in vitro culture of neonatal mouse SSCs generates male germline stem (GS) cells (Kanatsu-Shinohara et al., 2003) and embryonic stem (ES)-like cells (Kanatsu-Shinohara et al., 2004a). GS cells maintain their germ cell character with botryoidally aggregated colonies and contribute to the germ cell lineage (Kanatsu-Shinohara et al., 2003). In contrast, tightly packed 3-dimensional colonies are observed on ES-like cells. These type of cells also express pluripotent stem cell markers and contribute to various tissues including germ cell lineage in chimeric mice (Kanatsu-Shinohara et al., 2004a) (Ko et al., 2009). Consistent with these reports, studies on the epigenetic features of mouse SSCs and progenitor cells revealed the plasticity of these cells. SSCs have a characteristic closely to the incomplete reprogrammed induced pluripotent stem cells (iPSc) (Liu et al., 2016). Moreover, genetically modified cultured SSCs can be produced transgenic animals (Kanatsu-Shinohara et al., 2008). This suggests that cultured SSCs could be a useful tool for making genetic modifications in animals through gene targeting and genome editing technologies.

In domestic species, gonocytes and SSCs isolated from neonatal and immature bovine testes can be maintained in culture (Aponte et al., 2006), (Izadyar et al., 2002), (Fujihara et al., 2011), (Oatley et al., 2016), (Sahare et al., 2016). However, there are limited reports about a culture system that can efficiently establish GS cell from livestock species. Most of the culture media for maintaining self renewal and proliferation of SSCs are contain serum as a component of culture media. Serum stimulates cell proliferation, but some undefined components in the serum cause inappropriate and detrimental effects on specific cells (Barnes and Sato, 1980) and lead cell differentiation of SSCs. It has been reported that mouse SSCs can be cultured under a serum free condition (Kanatsu-Shinohara

et al., 2005), but there are no reports about a serum- and feeder-free culture system in bovine SSCs. Recent works reported that knockout serum replacement (KSR) in culture medium can maintain the proliferation of gonocytes and significantly reduce the concentration of serum to 1% in the medium (Sahare et al., 2016).

Glial cell line-derived neurotrophic factor (GDNF) is an essential factor for the maintenance and proliferation of mouse SSCs (Kubota et al., 2004) (Takashima et al., 2015) or bovine gonocytes (Sahare et al., 2015). Moreover, leukemia inhibitory factor (LIF) are also reported as supporting factor in the generation of mouse multipotent germ line stem cells from mouse SSCs (Guan et al., 2006). Recent study showed that appearing colonies in culture of bovine gonocytes culture have a morphology like mouse ES cells (Sahare et al., 2015). However, there are no further reports whether their cell characteristics and gene expression profiles were not well defined.

Even though there are several studies about the establishment of stem-cell like cell lines from mouse SSCs, however, there are limited studies for germline potency of SSCs in mammalian species other than mice. This remain a challenge to study about this unique stem cells and their transition mechanism from SSCs to pluripotent stem cells. In this report, a culture system for bovine SSCs in a under serum free condition were developed. The SSCs derived cell lines shared pluripotent stem cell characteristics, but also had germ cell characteristics.

3.2. Materials and methods

3.2.1. Isolation of SSCs from bovine testes

SSCs were isolated by sequential enzymatic digestion methods as previously reported (Fujihara et al., 2011) with minor modifications. Briefly, the testes were collected from 3 months old Japanese Black cattle. The collected testes were placed immediately into a tube and immersed in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (GIBCO BRL Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU mL^{-1} penicillin

(Sigma-Aldrich, St. Louis, MO, USA), 50 mg mL^{-1} streptomycin (Sigma-Aldrich), 40 mg mL^{-1} gentamicin sulfate (Sigma-Aldrich) and 15 mM HEPES (Wako, Osaka, Japan). The testes were transported to the laboratory at 4°C to 10°C within 24 h. To prepare cell suspensions, we minced the decapsulated testicular tissues and treated with a three-step enzymatic digestion method. The cells were incubated with the first collagenase solution for 50 min at 37°C, then washed three times with DMEM/F12. This was followed by the incubation with a second collagenase solution for 50 min at 37°C, after which the cells were washed again three times with DMEM/F12. The obtained suspensions were centrifuged, and the resulting cell pellets were incubated with a third collagenase solution for only 10 min at 37°C, washed three times and filtered through a 50-μm nylon mesh.

Percoll gradient centrifugation was used for the enrichment of SSCs. Percoll gradient of 60%, 40%, and 20% Percoll (GE Healthcare Life Sciences, Sweden) was used as previously reported (Fujihara et al., 2011) with minor modifications. Cell suspensions were loaded on top of the Percoll gradients and centrifuged at 3,000 rpm for 30 min. After centrifugation, different cell fractions were observed. The germ-cell fraction was collected from the 20 ~ 40% Percoll fraction.

3.2.2. In vitro culture of SSCs

Collected SSCs were cultured at 5 x 10^4 cells/cm^2 on poly-L-lysine (0.01%)-precoated wells in 24-well plates with basic media containing DMEM/F12, 100 μg/mL penicillin (Sigma-Aldrich), 50 μg/mL streptomycin (Sigma-Aldrich), 40 μg/mL gentamycin sulfate (Sigma-Aldrich), 10 μg/mL apotransferrin (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), 110 μg/mL sodium pyruvate (Sigma-Aldrich), 0.015% (v/v) sodium lactate (Sigma-Aldrich), 0.1% (v/v) non-essential amino acid solution (GIBCO BRL Invitrogen), and 0.01 mM β-mercaptoethanol (Wako). In the initial culture, the basic media were supplemented with 15% knockout serum replacement (KSR), 1% FBS and 20

ng/ml GDNF. After the first passage, the media were replaced with DMEM/F12 supplemented with 20% KSR and growth factors. Different growth factors—GDNF, bLIF, or both in conjunction—were added to find the optimal culture conditions for SSCs from immature testes. To establish cell lines, we employed a combination of GDNF and bLIF. The culture media were changed every 3 days.

3.2.3. Immunocytochemical analysis of cultured SSCs

Immunocytochemical analysis on freshly isolated or cultured cells was performed as described previously (Kim et al., 2014). Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized, blocked, and incubated with the appropriate primary and secondary antibodies indicated below.

The following antibodies and dilutions were used: anti-DBA conjugated with fluorescein isothiocyanate (FITC) (1: 200; Vector Laboratories, Burlingame, CA, USA), anti-UCHL-1 (PGP 9.5; 1:200; Santa Cruz Biotechnology), anti-SOX2 (1:200; Santa Cruz Biotechnology), and anti-GFAP (GFAP, 1 : 100; DAKO A/S). All primary antibodies were incubated with the specimens overnight at 4°C. Then, the cells were washed three times with TBST (Sigma), incubated with the appropriate secondary antibodies—either Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) or Alexa Fluor 594 rabbit anti-goat (Invitrogen)—for 1 h at room temperature, and washed three more times with TBST. The nuclei were counterstained with Hoechst 33342 (Sigma), and the specimens were mounted in 50% glycerol. Samples were observed under an immunofluorescence microscope (BX 50; Olympus, Tokyo, Japan).

3.2.4. RT-PCR

RNA was isolated from either freshly isolated testicular cells or cultured cells using TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. cDNA synthesis was performed using 1 μg of total RNA per 20 μL of PCR reaction mixture. Briefly, oligo(dT) primers were added to the isolated RNA and incubated for 5

min at 65°C. ReverTra Ace (Toyobo, Tokyo, Japan) was added to the RNA mixture for the reverse transcriptase reaction. The samples were incubated for 60 min at 42°C followed by 5 min at 99°C. The collected cDNA was stored at -20°C or immediately used for PCR. PCR amplification was performed using 1 μL of cDNA per 20 μL of PCR reaction mixture containing 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 x PCR buffer, 5 pmol of each primer and 1 U of Taq DNA polymerase (ExTaq; TaKaRa, Tokyo, Japan). The primer sequences used for the amplification of specific genes are listed in Table 2-1. The PCR products were separated and visualized on 2% (w/v) agarose gels containing ethidium bromide.

3.2.5. Karyotype analysis

Karyotype analysis was performed using a previously described protocol (Campos et al., 2009). Cultured cells were incubated in 0.1 μg/ml KaryoMAX Colcemid Solution (Invitrogen) for 3 hours. The cells were dissociated to a single-cell suspension using 0.25% trypsin-EDTA solution. Metaphase spreads were prepared by incubating single-cell suspensions in a prewarmed (37°C) hypotonic solution (KCl 75 mM) for 15 minutes and fixed in 3:1 (methanol (Wako):glacial acetic acid (Sigma)) fixative solutions overnight at 4°C. Chromosomes were stained with VECTASHIELD mounting medium with DAPI (Vector) and observed under an immunofluorescence microscope (BX 50; Olympus, Tokyo, Japan). Thirty metaphase spreads from three independent cell lines were counted.

3.2.6. In vitro differentiation analysis

To analyze in vitro differentiation potential of established cell lines, cell colonies were disassociated by 0.25% trypsin and 0.5 mM EDTA and then to obtain embryoid bodies (EB), 1.000-2.000 cells were transferred to MPC-treated round-bottom dishes (Nunc) in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) containing 15% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, NEAA, 0.1 mM 2-mercaptoethanol, penicillin, and streptomycin. Three days later, formation of embryoid bodies was confirmed, transferred to gelatin-coated culture dishes and further cultured for 9 days.

3.2.7. Aggregation of SSC-derived cell lines with bovine embryos

Bovine oocytes were fertilized in vitro as described elsewhere (Ikeda et al., 2017) and fertilized egg after 20 hours post-insemination (hpi) were treated with pronase solution (0.5% [w/v] in PBS) to remove the zona pellucida. The zona-free fertilized eggs were individually allocated to a well-of-the-well system (Vajta et al., 2008) using the LinKID micro25 culture dish (Dai Nippon Printing) with 50 μL of modified synthetic oviduct fluid

(mSOF) and cultured until 70 hpi. At 70 hpi, only embryos that had developed to the 8-cell to 16-cell stages were collected, and the culture medium was replaced with mSOF medium containing 5% (v/v) KSR, bLIF-conditioned medium (1:1000 dilution), and 20 ng/ml GDNF. SSC-derived cell lines were cultured on 35 mm poly-l-lysin pre-coated dish and tagged with 0.1 μL of Vybrant CFDA SE cell tracer dye (Invitrogen) according to the manufacturer's instructions. Subsequently, cell lines were mixed with zona-free embryos at the 8-cell to 16-cell stages. After four to five days after culture, the tracer dye on cells were observed under a fluorescence microscope. For subsequent examination, embryos were fixed with 4% PFA, prepared on slides, and counterstained with Hoechst 33342 (Sigma).

3.3. Results

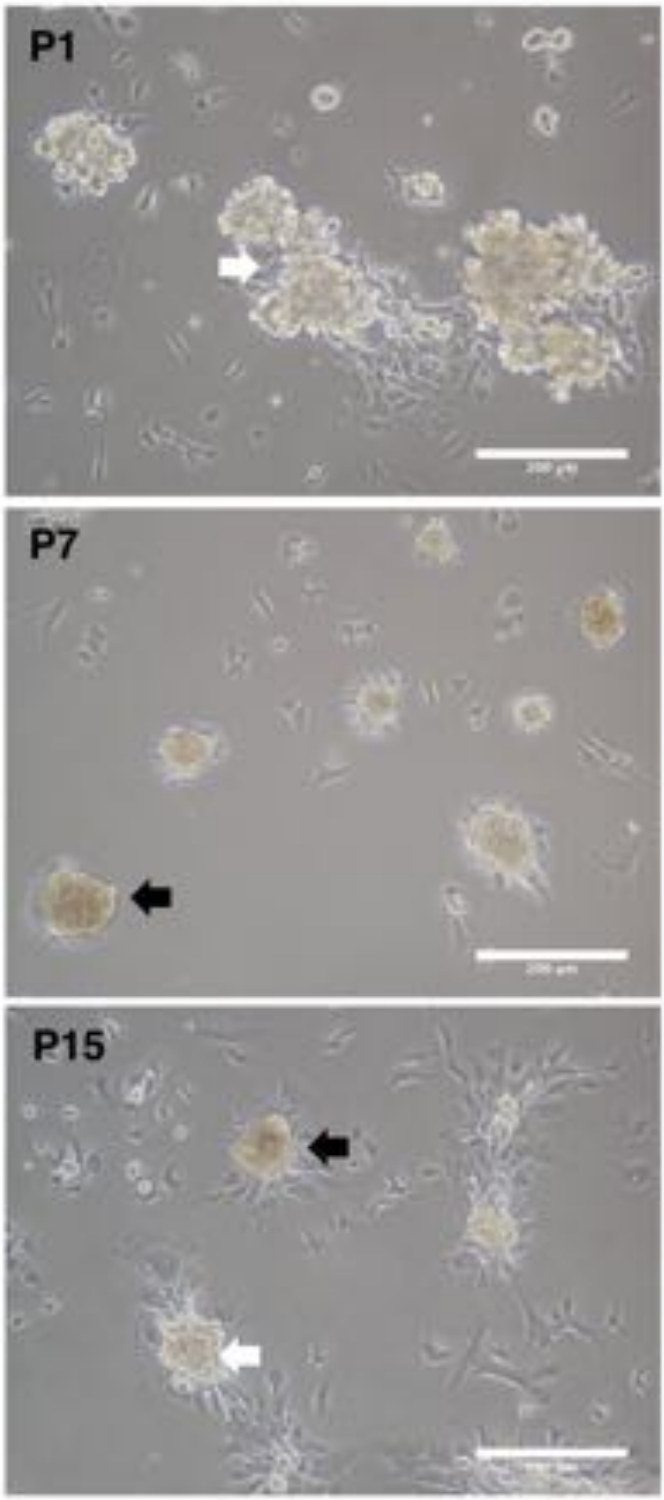
3.3.1. Derivation of cell lines

SSCs were isolated from 3-month-old immature cattle. Isolated cells were cultured in medium containing both 15% KSR and 1% FBS and supplemented with GDNF for the initial culture. After 5 to 7 days in primary culture, the cultured cells formed botryoidally aggregated multicellular colonies. After the first passage, the culture medium was changed to DMEM/F12 medium supplemented with 20% KSR, and the cells were passaged every 5 days. In the later passages (passages 7 and 8), the cultures were passaged every 2 to 3 days owing to robust proliferation of SSCs. The SSC cell lines formed botryoidally aggregated colonies in primary culture (Figure 3.1 A, white arrow) and gradually changed their morphology to become dome-shaped ES cell-like colonies after several passages (Figure 3.1 A, black arrow).

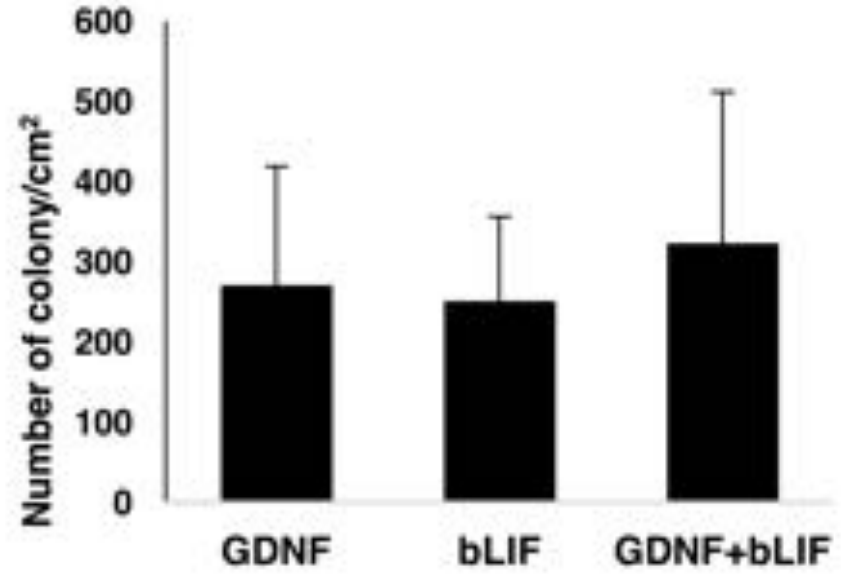
The effects of different growth factors on the proliferation of SSCs were also **tested. We compared the effect of KSR media supplemented with GDNF only, bLIF only,** and a combination of GDNF and bLIF on the number of colonies formed (Figure 3.1 B) and the total number of cells (Figure 3.1 B, lower panel). The results

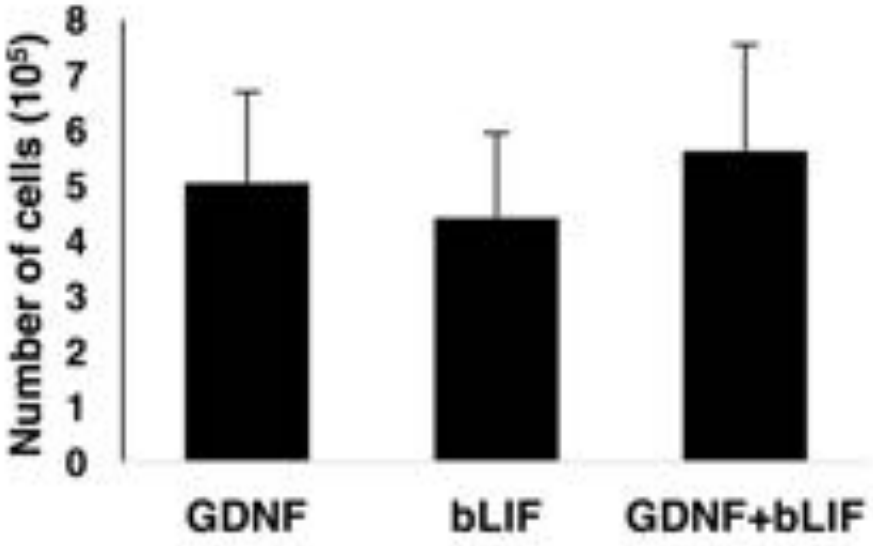
suggested that there were no significant differences among different culture conditions (Figure 3.1 B). However, RT-PCR results revealed that the colonies cultured in the presence of bLIF or GDNF and bLIF strongly expressed *UCHL-1* after long-term culture (Figure 3.1 C).

A



B


600
500
400
300
200
100
0
Number of colony/cm²
GDNF
bLIF
GDNF+bLIF


8
7
6
5
4
3
2
1
0
Number of cells (10⁵)
GDNF
bLIF
GDNF+bLIF

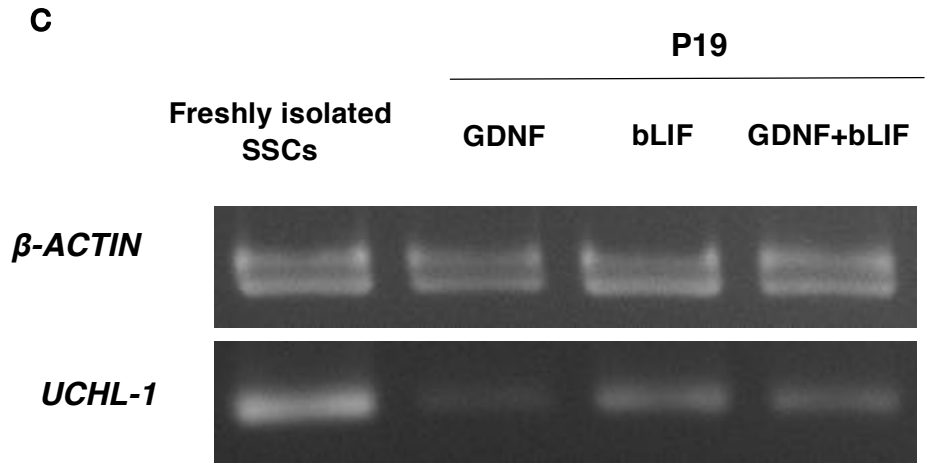
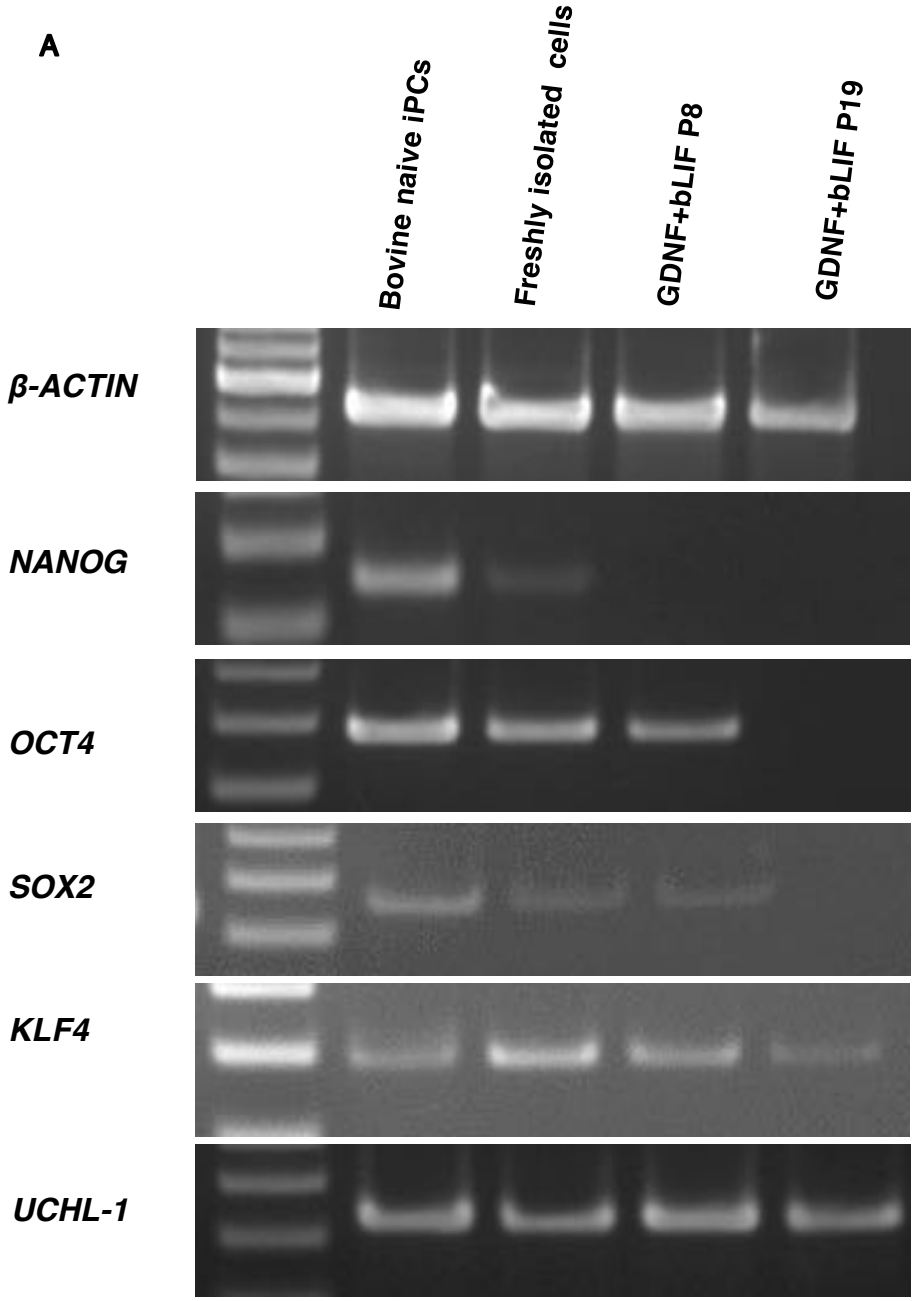


Figure 3-1. SSCs isolated from immature testes cultured in medium containing GDNF, bLIF, or both GDNF and bLIF.

Morphology of the colonies cultured in medium containing GDNF and bLIF. White arrows indicate botryoidally aggregated colonies resembling mouse GS cells, and black arrows indicate tightly packed 3-dimensional colonies resembling ES-like cells (A). Number of colonies/cm^2 after the first passage (P1) and total number of cells after P1 (B); RT-PCR analysis of UCHL-1 at P19 in colonies cultured under different culture conditions (C). Scale bars represent 200 μm.


A
Bovine naive iPCs
Freshly isolated cells
GDNF+bLIF P8
GDNF+bLIF P19
β-ACTIN
NANOG
OCT4
SOX2
KLF4
UCHL-1

B

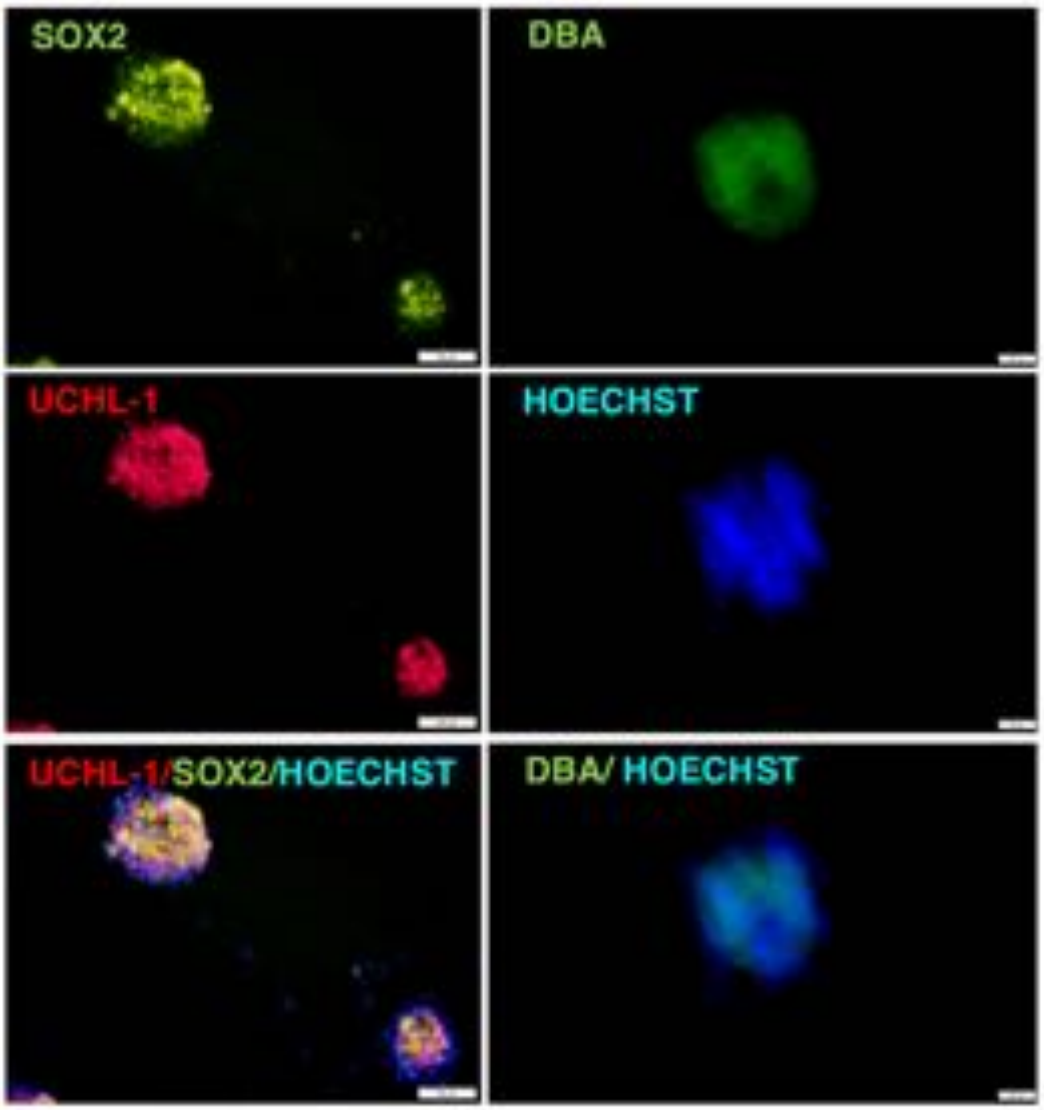


Figure 3-2. Characterization of cell lines from immature testis.

SSCs cultured in medium containing GDNF and bLIF. RT-PCR analysis of NANOG, OCT3/4, KLF4, SOX2 (stem cell markers) and UCHL-1 (a germ cell marker) (A); immunocytochemical analysis of the expression of SOX-2, UCHL-1, and DBA (B).

3.3.2. Characterization of SSCs-derived cell lines

RT-PCR analysis was performed on freshly isolated SSCs and 4 cultured cell lines at passage 8 and passage 19 (Figure 3.2 A). Freshly isolated SSCs express pluripotency-associated genes (*Nanog*, *OCT4*, *SOX2*, and *KLF4*) as well as the germ cell marker *UCHL*-1. Cell lines also expressed *OCT4*, *SOX2* and *KLF4* and *UCHL*-1 at passage 8. At the passage 19, however, only *KLF4* and *UCHL*-1 were detected (Figure 3.2 A). Immunofluorescent analysis was performed on the cell lines and detected a pluripotent marker (SOX2) and germ cell markers (UCHL-1 and DBA) (Figure 3.2 B).

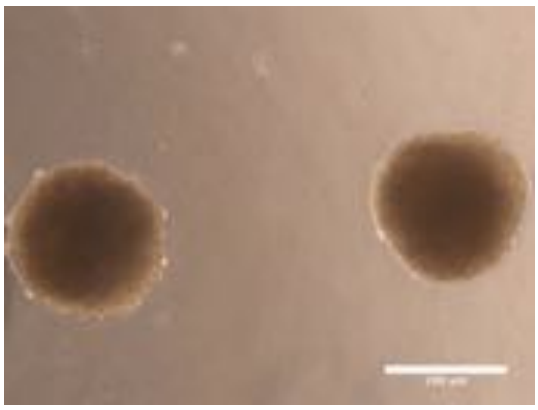
3.3.3. Differentiation potential of SSCs-derived cell lines

The developmental potential of SSC lines in vitro were examined by in vitro differentiation analysis on low adhesion U-shape dishes. All examined cell lines were able to form EB within 3 days of culture (Figure 3.3 A). Next, the EB were also differentiated into neuron-like cells as confirmed by the expression of glial fibrillary acidic protein (GFAP) by immunocytochemical analysis (Figure 3.3 B). Moreover, RT-PCR analysis showed that SSCs cell lines were expressed VIMENTIN (ectoderm marker), BMP4 (mesoderm marker) , and GATA6 (endoderm marker) (Figure 3.3 C).

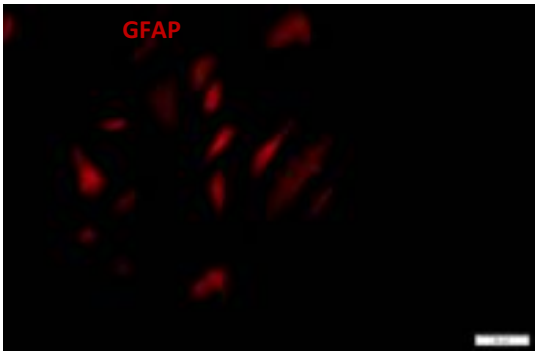
3.3.4. Karyotype analysis of SSCs-derived cell lines

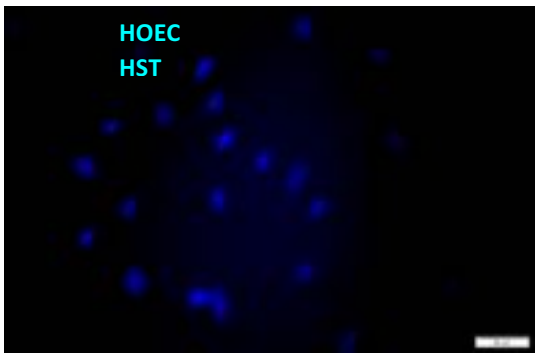
Karyotype analysis also showed normal metaphase spreads and chromosome number (2n=60) in SSC cell lines (93,3%, 28 out of 30 spreads), while 6.66% had 59 chromosomes in cell lines (Figure 3.4 A-B).

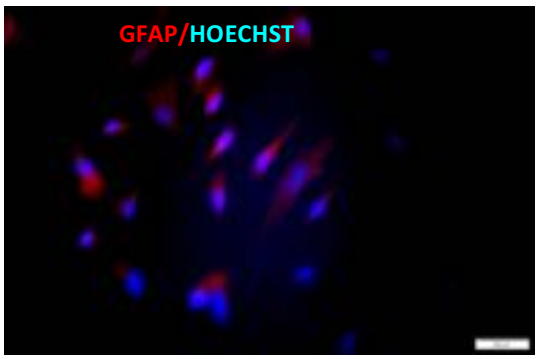
A



B







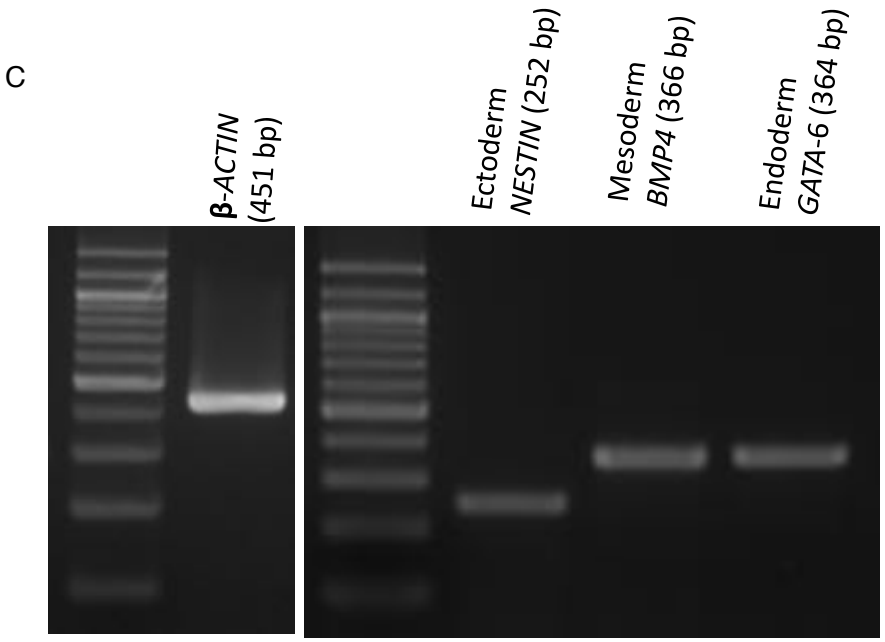


Figure 3-3. In vitro differentiation potential of SSCs derived cell lines.

Studied cell lines formed embryonic bodies (EBs) after cultured for 3 days using low-adhesion culture dishes (A), the expression of glial fibrillary acidic protein (GFAP) by immunocytochemical analysis (B) and ectoderm (*NESTIN*), mesoderm (*BMP4*), and endoderm (GATA-6) markers by RT PCR (C) of differentiating EBs after 9 days of culture (C).

A

B

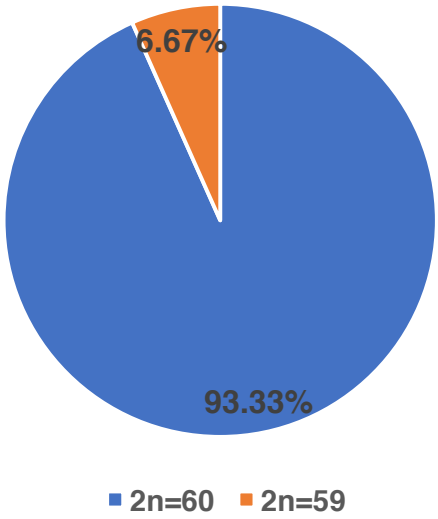


Figure 3-4. Karyotyping image of cells from immature and adult testis.

SSC-derived cell lines at passages 12 and 14 (A). Percentage of cells with normal chromosome number (n=30) (B).

3.3.5. Stem cell potential of established SSC cell lines

To examine the stem cell potential of bovine SSC cell lines, the cell lines were tagged with a long-term fluorescent tracer CFDA SE cell tracer kit and allowed them to aggregate into embryos at the 8- to 16-cell stages (Figure 3.5). Two different cell lines, which were from immature testes and from adult testes, were tested. Cell lines from immature testes were incorporated into the ICM of blastocysts (26.67%) (Table 3.2), whereas cell lines from adult testes only scattered around embryos or formed cell clumps outside of embryos (Figure 3.5), and a few of them incorporated specifically into the trofectoderm (TE) region of embryos (15.38%) (Table 3.2). In contrast, neither freshly isolated cells nor bovine embryonic fibroblasts (BEFs) were incorporated into the embryos. Bovine naïve-type iPS cells, which were previously established (Kawaguchi et al., 2015), were used as positive control. These cells were highly incorporated into the embryos (14.29% in the ICM only and 85.71% in the ICM and TE) (Table 3.2).

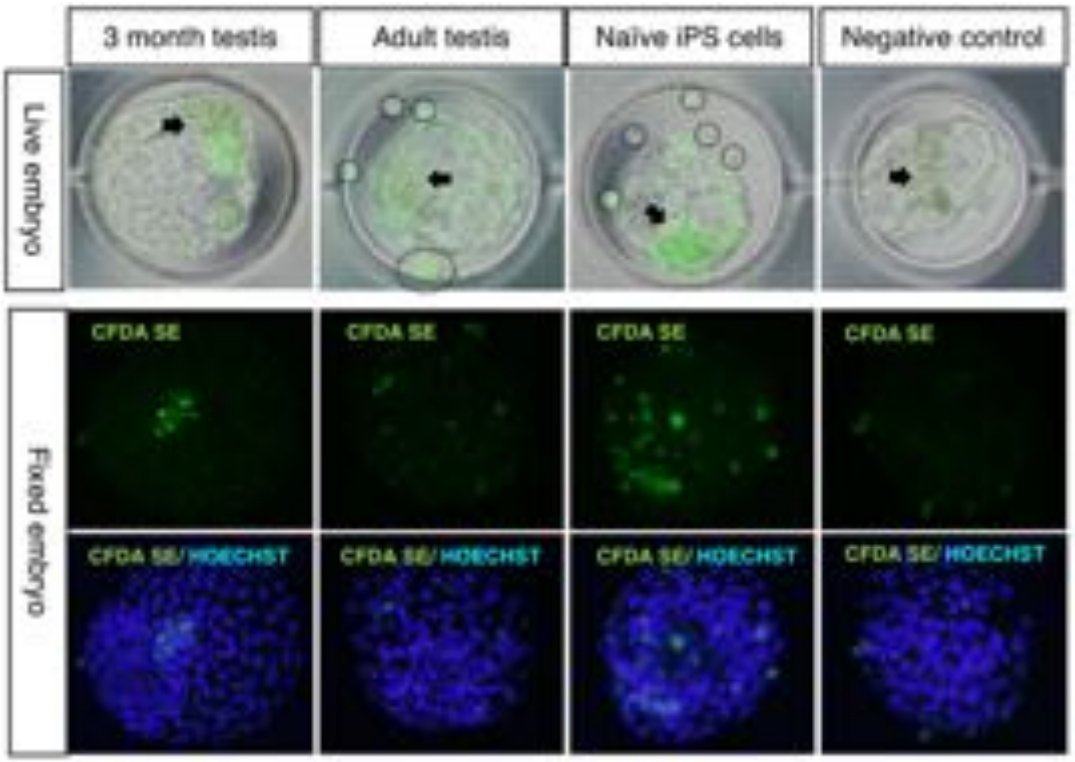


Figure 3-5. Contribution of SSC-derived cell lines from both immature and adult testes into bovine embryos.

SSC-derived cell lines were tagged with CFDA SE cell tracer and were aggregated with 8- to 16-cell embryos; naïve-type bovine iPS cells and embryos only were used as positive and negative controls, respectively. Black dashed circles indicated non-integrated cells; black arrows indicate the inner cell mass (ICM).

Table 3-1. Aggregation of SSCs-derived cell lines into in vitro fertilized bovine embryos.

Donor cells	No. of aggregated embryos	No. of blastocysts developed	Chimeric blastocysts		
			No. integrated to ICM (%)	No. integrated to both ICM and TE (%)	No. integrated to TE (%)
3 month testes	37	15 (40.54)	4 (26.67)	0 (0.00)	0 (0.00)
Adult testes	35	13 (37.14)	0 (0.00)	0 (0.00)	2 (15.38)
Freshly isolated cells	13	5 (38.46)	0 (0.00)	0 (0.00)	0 (0.00)
BEF	19	8 (42.11)	0 (0.00)	0 (0.00)	0 (0.00)
Bovine naive iPSCs	14	7 (50.00)	1 (14.29)	6 (85.71)	0 (0.00)
Embryo only	19	10 (52.63)	0 (0.00)	0 (0.00)	0 (0.00)

3.1 Discussion

Experimental results showed that SSCs isolated from 3 months old testes can give rise to cell lines under serum-free and feeder-free conditions. When SSCs were kept in culture containing GDNF and FBS, growing SSCs colonies were observed. However, we also observe proliferation of somatic cells overgrowing colonies after few passages. By contrast, when SSCs were cultured under serum-free conditions in the presence of KSR medium containing both GDNF and bLIF, SSC colonies were formed and maintained for a long period.

SSC colonies derived from immature testes were identified by their botryoidally aggregated cell morphology (Figure 3.1 A) and then transformed to tightly packed 3-dimensional colonies similar to mouse ES cells after a month of culture (Figure 3.1 A). These ES cell-like colonies were maintained for only 2 months (11 passages); however, the botryoidally aggregated colonies were more stable and could be maintained for more than 3 months under serum-free culture conditions. Mouse SSCs isolated from neonatal mouse testes start to form ES-like colonies within 4-7 weeks after the initial culture (Kanatsu-Shinohara et al., 2004a) and can subsequently be cultured under ES cell culture conditions while maintaining their ES-cell like properties (Kanatsu-Shinohara et al., 2004a). ES-like bovine SSCs colonies were cultured on STO feeder cells, which is the same culture condition used for previously established bovine naïve-type iPS cells (Kawaguchi et al., 2015); however, SSCs colonies could not be maintained under this condition.

The established cell lines from immature testes had mouse ES-cell like morphology in serum-free medium supplemented with GDNF and bLIF, and they expressed both germ-cell-specific (*UCHL-1* and *DBA*) and pluripotent-stem- cell-specific markers (*OCT4*, *SOX2*, *KLF4*). Consistent with these results, other studies have reported that in vitro culture of gonocytes from neonatal bovine testes yields ES cell-like colonies (Sahare et al., 2015) that express cell surface markers of ES cells (Li et al., 2016a). They also differentiated in vitro into three germ layers confirmed by the expression of ectoderm (NESTIN), mesoderm

(BMP4), and endoderm (GATA-6) markers by RT-PCR and neuron like-cells confirmed by the expression of glial fibrillary acidic protein (GFAP) by immunocytochemical analysis.

To examine the stem cell potential of the established cell lines, we co-cultured three different cell lines with 8- to 16-cell embryos produced by in vitro fertilization of bovine eggs. SSCs-derived cell lines were incorporated into the ICM region in 26.67% of blastocysts (Table 3.2 and Figure 3.5); however, none of the freshly isolated SSCs could be incorporated into blastocysts. In contrast, the contribution of cell lines from adult testes was limited, consistent with a study showing that the contribution of mouse SSC-derived cell lines to chimeras is dependent on the age of the testes (Azizi et al., 2016). In previous reports (Kawaguchi et al., 2015), established naïve-type bovine iPS cells were abundantly incorporated into both the ICM and TE regions of blastocysts after aggregation with 8-cell and 16-cell embryos. Taken together, the evidence shows that cells of cell lines derived from immature testes have partial stem cell potential, probably because of a heterogeneous stem cell population in the established cell lines or a cell population with both stem-cell and germ-cell characteristics, but those from adult testis have narrower limits on their potential as stem cells.

In conclusions, a culture system was developed for SSCs isolated from immature bovine testes. The established cell lines are maintained in serum-free culture conditions in the presence of GDNF and bLIF. These cell lines have ES-like cell morphology, express pluripotent-stem-cell- and germ-cell-specific markers, and incorporated to the ICM when they aggregated with bovine embryo. Further studies are necessary to examine the ability of cell lines to contribute to chimeric fetuses and contribute to spermatogenesis through their germ-cell potential in bovine testes.

Chapter 4

General Summary

4.1. Summary

During the development of animal biotechnology, numerous attempts are conducted to establish cell lines from male undifferentiated germ (MUG) cells consisting of spermatogonial stem cells (SSCs). It has been reported that mouse neonatal SSCs can give rise to embryonic stem like (ES-like) cells, and contribute to chimeric embryos including germ-cell lineage. Moreover, genetically modified cultured SSCs allow the production of transgenic animals. This suggests that cultured SSCs can be a useful tool for introducing genetic modification through gene targeting and genome editing technologies. The problem is that SSCs are a small population of germ cell in the testis that are difficult to isolate from their differentiated germ cell population. Furthermore, the establishment of cell lines from SSCs seems to be dependent on species and the age of animals. To our knowledge, there are no reports about the culture systems of SSCs isolated from adult livestock animals. It has been shown that bovine gonocytes (an embryonic precursor of SSCs) and SSCs isolated from neonatal to 3 months old animals can be maintained in the culture for long-term. However, when the neonatal culture system was applied to a subset of bovine germ cells isolated from adult testes, they could not be maintained for a long time. Because of their great promise for biotechnology and agriculture, there are lots of interests to derive cell lines from male undifferentiated germ cell in domestic species from both adult and immature testes.

Adult undifferentiated germ cell lines, which express markers of germ cell lineage, were successfully established in this study as descried in chapter 2. Undifferentiated germ cells are rare in adult seminiferous tubules, the isolation of these cells must be followed by enrichment procedures. Cell suspensions were prepared from adult bovine testes followed by two enrichment steps. Percoll gradient centrifugation (first enrichment) was followed by plating the cell suspensions on gelatin-coated dishes (second enrichment). The enriched cells were cultured for the first 10 passages in the medium containing BIO and

subsequently the cultures were maintained in the medium containing GDNF (Figure 4-1). Typical mouse male germ stem (GS) like cell colonies were formed under these culture conditions as recognized by their botryoidally morphology. All cell lines expressed germ-cell-specific markers such as UCHL-1, DBA, and GFRα-1 and pluripotent stem-cell-specific markers *OCT4*, *SOX2*, and *KLF4*. Germ cells were cultured for about 3 months under these conditions. It suggests that adult bovine undifferentiated germ cells can be cultured in vitro for long-term and resemble mouse GS cell line confirmed by the expression of germ-cell-specific markers, pluripotent stem-cell-specific markers, and the morphology of colonies. This method was successfully applied to culture germ cells isolated from 17 years old bovine testis, but only for short-term. The reason why the bMUG cells did not proliferate for a long-term is that 17 years old testis might have only limited number of germ cells under "quiescent" state after a long-term break of semen collections.

In chapter 3, undifferentiated germ cell consisting of SSCs isolated from 3 months old testes can give rise to germ-cell lines under serum-free culture conditions. When SSCs were kept in culture in the presence of GDNF and fetal bovine serum (FBS), they formed colonies. However, testicular somatic cells also proliferated well and overgrew the cultures after few passages. In contrast, when SSCs were cultured under serum-free conditions in the presence of knockout serum replacement (KSR) medium containing both GDNF and bLIF, somatic cells did not grow, and the colonies were formed and maintained for a long-term. During the early stages of culture, the colonies were identified by their botryoidally morphology. After about one month of the culture, tightly packed 3-dimensional colonies like as mouse embryonic stem (ES) cells were observed. These ES cell-like colonies were maintained for at least 2 months. However, the grape-like shaped colonies were maintained for more than 3 months under serum-free culture conditions. The expression of both germ-cell- specific markers (UCHL-1 and DBA), and pluripotent stem-cell-specific markers (*OCT4*, *SOX2*, *KLF4*) were also confirmed when predominant ES-like colonies were

present in the culture. When in vitro differentiation analysis was performed, cell lines formed embryonic bodies (EBs). After EBs were cultured for another 9 days, they differentiated into three germ layers confirmed by the expression of ectoderm (*NESTIN*), mesoderm (*BMP4*), and endoderm (*GATA*-6) markers by RT-PCR. Immunofluorescense analysis suggested that the cell lines differentiated into neuron-like cells confirmed by the expression of GFAP. These results indicate that serum-free medium supplemented by GDNF and bLIF support the establishment of cell lines exhibiting characteristic of both germ cells and stem cells.

The stem cell potential of the established cell lines both of immature and adult testes were examined by aggregation using three different cell lines with 8- to 16-cell stage embryos. Cell lines from immature testes were incorporated into the ICM region in 26.67% of blastocysts. In contrast, the contribution of cell lines from adult testes was limited. Taken together, the evidence shows that cells of cell lines derived from immature testes have a partial stem cell potential, but those from adult testis have strong characteristic as germ cell lines and a weak potential as stem cells.

4.2. Conclusions

A long-term culture system was developed for undifferentiated germ cells consisting of SSCs isolated either from immature or adult testis. Cell lines from adult testes are established in a low serum concentration media in the presence of BIO and GDNF. The cell lines have characteristics resembling to previously reported mouse male germ cell lines as confirmed by botryoidally morphology, the expression of germ cell specific markers, and pluripotent stem cell markers. While cell lines established from immature testes are maintained in serum-free culture conditions in the presence of GDNF and bLIF. These cell lines have ES-like cell morphology, express pluripotent stem cell and germ cell specific markers at protein and mRNA levels, and incorporated into the ICM region of blastocyst stage embryos.

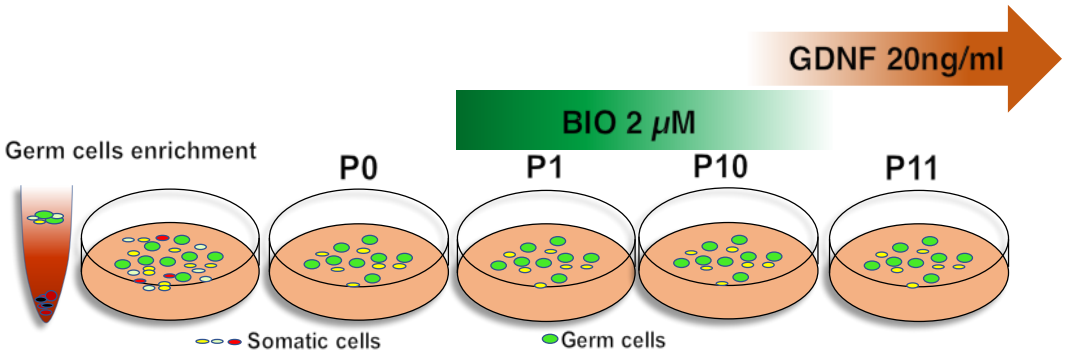


Figure 4-1. Schematic procedure for a long term culture of adult bovine undifferentiated germ cells.

After germ cells were isolated by three-step enzymatic digestions, then undifferentiated germ cells were enriched by Percoll gradient centrifugation and differential plating on gelatin coated dish. Undifferentiated germ cells were cultured in medium DMEM/F12+15%KSR+1%FBS. GDNF was added during the initial culture and replaced by BIO during the period from the first passage to the passage 10, at the passage 11 BIO was finally removed again and replaced by GDNF.

4.3. Future directions

Different culture systems for bovine undifferentiated germ cells consisting SSCs isolated from both immature and adult testes were established in this study. This finding demonstrates that the feasibility of SSC culture in domestic species, which could facilitate progress in research related to transgenic animal production, genome editing technology for the improvement of livestock production or conservation of endangered species. However, further investigations are still necessary for determining the ability of cell lines to contribute to spermiogenesis and chimeric fetuses, and subsequently use these cell lines for genome editing technology.

References

Aponte, P.M., Soda, T., van de Kant, H.J.G., and de Rooij, D.G. (2006). Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. Theriogenology *65*, 1828–1847.

Azizi, H., Conrad, S., Hinz, U., Asgari, B., Nanus, D., Peterziel, H., Hajizadeh Moghaddam, A., Baharvand, H., and Skutella, T. (2016). Derivation of Pluripotent Cells from Mouse SSCs Seems to Be Age Dependent. Stem Cells Int. *2016*, https://doi.org/10.1155/2016/8216312.

Barnes, D., and Sato, G. (1980). Serum-free cell culture: a unifying approach. Cell *22*, 649–655.

Cai, H., Wu, J.-Y., An, X.-L., Zhao, X.-X., Wang, Z.-Z., Tang, B., Yue, Z.-P., Li, Z.-Y., and Zhang, X.-M. (2016). Enrichment and culture of spermatogonia from cryopreserved adult bovine testis tissue. Anim. Reprod. Sci. *166*, 109–115.

Campos, P.B., Sartore, R.C., Abdalla, S.N., and Rehen, S.K. (2009). Chromosomal spread preparation of human embryonic stem cells for karyotyping. J. Vis. Exp. JoVE.

Chakraborty, P., Buaas, F.W., Sharma, M., Snyder, E., de Rooij, D.G., and Braun, R.E. (2014). LIN28A marks the spermatogonial progenitor population and regulates its cyclic expansion. Stem Cells Dayt. Ohio *32*, 860–873.

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Bühring, H.-J., Mattheus, U., Mack, A., et al. (2008). Generation of pluripotent stem cells from adult human testis. Nature *456*, 344–349.

Costa, G.M.J., Avelar, G.F., Rezende-Neto, J.V., Campos-Junior, P.H.A., Lacerda, S.M.S.N., Andrade, B.S.C., Thomé, R.G., Hofmann, M.-C., and Franca, L.R. (2012). Spermatogonial Stem Cell Markers and Niche in Equids. PLOS ONE *7*, e44091.

Culty, M. (2009). Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res. Part C Embryo Today Rev. *87*, 1–26.

Fujihara, M., Kim, S.-M., Minami, N., Yamada, M., and Imai, H. (2011). Characterization and in vitro culture of male germ cells from developing bovine testis. J. Reprod. Dev. *57*, 355–364.

Gassei, K., Ehmcke, J., and Schlatt, S. (2009). Efficient enrichment of undifferentiated GFR alpha 1+ spermatogonia from immature rat testis by magnetic activated cell sorting. Cell Tissue Res. *337*, 177–183.

Goel, S., Sugimoto, M., Minami, N., Yamada, M., Kume, S., and Imai, H. (2007). Identification, Isolation, and In Vitro Culture of Porcine Gonocytes. Biol. Reprod. *77*, 127–137.

Goel, S., Reddy, N., Mandal, S., Fujihara, M., Kim, S.-M., and Imai, H. (2010). Spermatogonia-specific proteins expressed in prepubertal buffalo (Bubalus bubalis) testis and their utilization for isolation and in vitro cultivation of spermatogonia. Theriogenology *74*, 1221–1232.

Guan, K., Nayernia, K., Maier, L.S., Wagner, S., Dressel, R., Lee, J.H., Nolte, J., Wolf, F., Li, M., Engel, W., et al. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. Nature *440*, 1199–1203.

Hasegawa, K., Namekawa, S.H., and Saga, Y. (2013). MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. Stem Cells Dayt. Ohio *31*, 2517–2527.

He, Z., Jiang, J., Kokkinaki, M., Golestaneh, N., Hofmann, M.-C., and Dym, M. (2008). Gdnf Upregulates c-Fos Transcription via the Ras/Erk1/2 Pathway to Promote Mouse Spermatogonial Stem Cell Proliferation. STEM CELLS *26*, 266–278.

Heidari, B., Rahmati-Ahmadabadi, M., Akhondi, M.M., Zarnani, A.H., Jeddi-Tehrani, M., Shirazi, A., Naderi, M.M., and Behzadi, B. (2012). Isolation, identification, and culture of goat spermatogonial stem cells using c-kit and PGP9.5 markers. J. Assist. Reprod. Genet. *29*, 1029–1038.

Herrid, M., Davey, R.J., and Hill, J.R. (2007). Characterization of germ cells from pre-pubertal bull calves in preparation for germ cell transplantation. Cell Tissue Res. *330*, 321–329.

Hilscher, B., Hilscher, W., Bülthoff-Ohnolz, B., Krämer, U., Birke, A., Pelzer, H., and Gauss, G. (1974). Kinetics of gametogenesis. Cell Tissue Res. *154*, 443–470.

Hofmann, M.-C. (2008). Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Mol. Cell. Endocrinol. *288*, 95–103.

Ikeda, S., Kawahara-Miki, R., Iwata, H., Sugimoto, M., and Kume, S. (2017). Role of methionine adenosyltransferase 2A in bovine preimplantation development and its associated genomic regions. Sci. Rep. *7*, 3800.

Ishii, K., Kanatsu-Shinohara, M., Toyokuni, S., and Shinohara, T. (2012). FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. Development *139*, 1734–1743.

Ishikura, Y., Yabuta, Y., Ohta, H., Hayashi, K., Nakamura, T., Okamoto, I., Yamamoto, T., Kurimoto, K., Shirane, K., Sasaki, H., et al. (2016). In Vitro Derivation and Propagation of Spermatogonial Stem Cell Activity from Mouse Pluripotent Stem Cells. Cell Rep. *17*, 2789–2804.

Izadyar, F., Spierenberg, G.T., Creemers, L.B., den Ouden, K., and de Rooij, D.G. (2002). Isolation and purification of type A spermatogonia from the bovine testis. Reprod. Camb. Engl. *124*, 85–94.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. *69*, 612–616.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., et al. (2004). Generation of Pluripotent Stem Cells from Neonatal Mouse Testis. Cell *119*, 1001–1012.

Kanatsu-Shinohara, M., Miki, H., Inoue, K., Ogonuki, N., Toyokuni, S., Ogura, A., and Shinohara, T. (2005). Long-Term Culture of Mouse Male Germline Stem Cells Under Serum-or Feeder-Free Conditions. Biol. Reprod. *72*, 985–991.

Kanatsu-Shinohara, M., Kato, M., Takehashi, M., Morimoto, H., Takashima, S., Chuma, S., Nakatsuji, N., Hirabayashi, M., and Shinohara, T. (2008). Production of transgenic rats via lentiviral transduction and xenogeneic transplantation of spermatogonial stem cells. Biol. Reprod. *79*, 1121–1128.

Kanatsu-Shinohara, M., Naoki, H., and Shinohara, T. (2016). Nonrandom Germline Transmission of Mouse Spermatogonial Stem Cells. Dev. Cell *38*, 248–261.

Kaucher, A.V., Oatley, M.J., and Oatley, J.M. (2012). NEUROG3 Is a Critical Downstream Effector for STAT3-Regulated Differentiation of Mammalian Stem and Progenitor Spermatogonia. Biol. Reprod. *86*. https://doi.org/10.1095/biolreprod.111.097386.

Kawaguchi, T., Tsukiyama, T., Kimura, K., Matsuyama, S., Minami, N., Yamada, M., and Imai, H. (2015). Generation of Naïve Bovine Induced Pluripotent Stem Cells Using PiggyBac Transposition of Doxycycline-Inducible Transcription Factors. PLOS ONE *10*, e0135403.

Kim, S.-M., Fujihara, M., Sahare, M., Minami, N., Yamada, M., and Imai, H. (2014). Effects of extracellular matrices and lectin Dolichos biflorus agglutinin on cell adhesion and self-renewal of bovine gonocytes cultured in vitro. Reprod. Fertil. Dev. *26*, 268–281.

Ko, K., Tapia, N., Wu, G., Kim, J.B., Bravo, M.J.A., Sasse, P., Glaser, T., Ruau, D., Han, D.W., Greber, B., et al. (2009). Induction of Pluripotency in Adult Unipotent Germline Stem Cells. Cell Stem Cell *5*, 87–96.

Kubota, H., Avarbock, M.R., and Brinster, R.L. (2003). Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc. Natl. Acad. Sci. *100*, 6487–6492.

Kubota, H., Avarbock, M.R., and Brinster, R.L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. U. S. A. *101*, 16489–16494.

Langenstroth, D., Kossack, N., Westernströer, B., Wistuba, J., Behr, R., Gromoll, J., and Schlatt, S. (2014). Separation of somatic and germ cells is required to establish primate spermatogonial cultures. Hum. Reprod. *29*, 2018–2031.

Lee, J., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Kimura, T., Nakano, T., Ogura, A., and Shinohara, T. (2007). Akt mediates self-renewal division of mouse spermatogonial stem cells. Development *134*, 1853–1859.

Lee, K.H., Lee, W.Y., Kim, J.H., Yoon, M.J., Kim, N.H., Kim, J.H., Uhm, S.J., Kim, D.H., Chung, H.J., and Song, H. (2013). Characterization of GFRα-1-positive and GFRα-1-negative spermatogonia in neonatal pig testis. Reprod. Domest. Anim. Zuchthyg. *48*, 954–960.

Li, B., Zhuang, M., Wu, C., Niu, B., Zhang, Z., Li, X., Wei, Z., Li, G., and Hua, J. (2016a). Bovine male germline stem-like cells cultured in serum- and feeder-free medium. Cytotechnology *68*, 2145–2157.

Li, C.-H., Yan, L.-Z., Ban, W.-Z., Tu, Q., Wu, Y., Wang, L., Bi, R., Ji, S., Ma, Y.-H., Nie, W.-H., et al. (2016b). Long-term propagation of tree shrew spermatogonial stem cells in culture and successful generation of transgenic offspring. Cell Res. https://doi.org/10.1038/cr.2016.156.

Liu, Y., Giannopoulou, E.G., Wen, D., Falciatori, I., Elemento, O., Allis, C.D., Rafii, S., and Seandel, M. (2016). Epigenetic profiles signify cell fate plasticity in unipotent spermatogonial stem and progenitor cells. Nat. Commun. *7*, 11275.

Mäkelä, J.-A., Toppari, J., Rivero-Müller, A., and Ventelä, S. (2014). Reconstruction of mouse testicular cellular microenvironments in long-term seminiferous tubule culture. PLOS ONE *9*, e90088.

Manku, G., and Culty, M. (2015). Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges. Reproduction *149*, R139–R157.

Meng, X., Lindahl, M., Hyvönen, M.E., Parvinen, M., Rooij, D.G. de, Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., et al. (2000). Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF. Science *287*, 1489–1493.

Nagano, M., Avarbock, M.R., and Brinster, R.L. (1999). Pattern and Kinetics of Mouse Donor Spermatogonial Stem Cell Colonization in Recipient Testes. Biol. Reprod. *60*, 1429–1436.

Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R.E., and Yoshida, S. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science *328*, 62–67.

Nasiri, Z., Hosseini, S.M., Hajian, M., Abedi, P., Bahadorani, M., Baharvand, H., and Nasr-Esfahani, M.H. (2012). Effects of different feeder layers on short-term culture of prepubertal bovine testicular germ cells in-vitro. Theriogenology *77*, 1519–1528.

Naughton, C.K., Jain, S., Strickland, A.M., Gupta, A., and Milbrandt, J. (2006). Glial Cell-Line Derived Neurotrophic Factor-Mediated RET Signaling Regulates Spermatogonial Stem Cell Fate. Biol. Reprod. *74*, 314–321.

Oakberg, E.F. (1971). Spermatogonial stem-cell renewal in the mouse. Anat. Rec. *169*, 515–531.

Oatley, J.M., and Brinster, R.L. (2012). The Germline Stem Cell Niche Unit in Mammalian Testes. Physiol. Rev. *92*, 577–595.

Oatley, J.M., Avila, D.M. de, Reeves, J.J., and McLean, D.J. (2004). Testis Tissue Explant Culture Supports Survival and Proliferation of Bovine Spermatogonial Stem Cells. Biol. Reprod. *70*, 625–631.

Oatley, J.M., Oatley, M.J., Avarbock, M.R., Tobias, J.W., and Brinster, R.L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development *136*, 1191–1199.

Oatley, M.J., Kaucher, A.V., Yang, Q.-E., Waqas, M.S., and Oatley, J.M. (2016). Conditions for Long-Term Culture of Cattle Undifferentiated Spermatogonia. Biol. Reprod. biolreprod.116.139832.

van Pelt, A.M., Morena, A.R., van Dissel-Emiliani, F.M., Boitani, C., Gaemers, I.C., de Rooij, D.G., and Stefanini, M. (1996). Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol. Reprod. *55*, 439–444.

Pramod, R.K., and Mitra, A. (2014). In vitro culture and characterization of spermatogonial stem cells on Sertoli cell feeder layer in goat (Capra hircus). J. Assist. Reprod. Genet. *31*, 993–1001.

Rooij, D. de (2001). Proliferation and differentiation of spermatogonial stem cells. Reproduction *121*, 347–354.

Rooij, D.G. de, Schrans-Stassen, B.H.G.J., Pelt, A.M.M. van, Shuttlesworth, G.A., Meistrich, M.L., Okabe, M., and Nishimune, Y. (2000). Regulation of the Differentiation of the Undifferentiated Spermatogonia. In The Testis, (Springer, New York, NY), pp. 43–54.

Sahare, M., Otomo, A., Komatsu, K., Minami, N., Yamada, M., and Imai, H. (2015). The role of signaling pathways on proliferation and self-renewal of cultured bovine primitive germ cells. Reprod. Med. Biol. *14*, 17–25.

Sahare, M., Kim, S.-M., Otomo, A., Komatsu, K., Minami, N., Yamada, M., and Imai, H. (2016). Factors supporting long-term culture of bovine male germ cells. Reprod. Fertil. Dev. *28*, 2039–2050.

Sato, H., Amagai, K., Shimizukawa, R., and Tamai, Y. (2009). Stable generation of serum- and feeder-free embryonic stem cell-derived mice with full germline-competency by using a GSK3 specific inhibitor. Genesis *47*, 414–422.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A.H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med. *10*, 55–63.

Sato, T., Katagiri, K., Gohbara, A., Inoue, K., Ogonuki, N., Ogura, A., Kubota, Y., and Ogawa, T. (2011). In vitro production of functional sperm in cultured neonatal mouse testes. Nature *471*, 504–507.

Seandel, M., James, D., Shmelkov, S.V., Falciatori, I., Kim, J., Chavala, S., Scherr, D.S., Zhang, F., Torres, R., Gale, N.W., et al. (2007). Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature *449*, 346–350.

Shinohara, T., Orwig, K.E., Avarbock, M.R., and Brinster, R.L. (2001). Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc. Natl. Acad. Sci. *98*, 6186–6191.

Song, H.-W., Bettegowda, A., Lake, B.B., Zhao, A.H., Skarbrevik, D., Babajanian, E., Sukhwani, M., Shum, E.Y., Phan, M.H., Plank, T.-D.M., et al. (2016). The Homeobox Transcription Factor RHOX10 Drives Mouse Spermatogonial Stem Cell Establishment. Cell Rep. *17*, 149–164.

Sun, F., Xu, Q., Zhao, D., and Chen, C.D. (2015). Id4 Marks Spermatogonial Stem Cells in the Mouse Testis. Sci. Rep. *5*, 17594.

Takase, H.M., and Nusse, R. (2016). Paracrine Wnt/β-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. Proc. Natl. Acad. Sci. U. S. A. *113*, E1489-1497.

Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Morimoto, H., Inoue, K., Ogonuki, N., Jijiwa, M., Takahashi, M., Ogura, A., and Shinohara, T. (2015). Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep. *4*, 489–502.

Tegelenbosch, R.A., and de Rooij, D.G. (1993). A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat. Res. *290*, 193–200.

Vajta, G., Korösi, T., Du, Y., Nakata, K., Ieda, S., Kuwayama, M., and Nagy, Z.P. (2008). The Well-of-the-Well system: an efficient approach to improve embryo development. Reprod. Biomed. Online *17*, 73–81.

Wang, S., Wang, X., Ma, L., Lin, X., Zhang, D., Li, Z., Wu, Y., Zheng, C., Feng, X., Liao, S., et al. (2016). Retinoic Acid Is Sufficient for the In Vitro Induction of Mouse Spermatocytes. Stem Cell Rep. *7*, 80–94.

Wang, X., Chen, T., Zhang, Y., Li, B., Xu, Q., and Song, C. (2015). Isolation and Culture of Pig Spermatogonial Stem Cells and Their in Vitro Differentiation into Neuron-Like Cells and Adipocytes. Int. J. Mol. Sci. *16*, 26333–26346.

Yeh, J.R., Zhang, X., and Nagano, M.C. (2012). Indirect Effects of Wnt3a/β-Catenin Signalling Support Mouse Spermatogonial Stem Cells In Vitro. PLoS ONE *7*, e40002.

Zheng, Y., Zhang, Y., Qu, R., He, Y., Tian, X., and Zeng, W. (2014). Spermatogonial stem cells from domestic animals: progress and prospects. Reproduction *147*, R65–R74.